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STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

V. THE INFLUENCE OF AMINO-ACIDS AND RELATED SUBSTANCES ON THE SYNTHESIS AND RATE OF ELIMINATION OF HIPPURIC ACID AFTER THE ADMINISTRATION OF BENZOATE.*

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The problem of the origin of the glycine available for hippuric acid synthesis after the administration of benzoic acid or its salts has been studied by many investigators with varying results. It should be borne in mind, in this connection, that all species of animals do not react similarly in response to the ingestion of benzoic acid. Thus the organisms of man (1), the rabbit (2), the goat (3, 4), and the pig (5, 6) have a store of readily available glycine as evidenced by the fact that they conjugate almost completely sublethal doses of benzoates and eliminate hippuric acid quantitatively. The dog (7), however, after benzoate ingestion, eliminates much free benzoic acid, a considerable amount of benzoic acid in conjugation with glycuronic acid, and less than 50 per cent of the ingested benzoic, in most cases, combined with glycine. Birds (8) eliminate dibenzoyl ornithine after the administration of benzoates and utilize ornithine rather than glycine as the detoxicating agent. The cause of these variations in the chemical mechanism by which benzoic acid is rendered less injurious to the organism is not known.

Analyses of the many attempts to discover the source of the glycine used by many species for detoxication of benzoic acid or its

*An abstract of a thesis submitted by Wendell H. Griffith in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate School of the University of Illinois.

salts would indicate that they may conveniently be divided into three groups: (a) studies of the alteration in the distribution of the nitrogen of the urine after benzoate ingestion; (b) the formation of hippuric acid from benzoic acid and possible precursors of glycine during perfusion of surviving organs, notably the liver and kidneys; and (c) the elimination of hippuric acid in the urine following the administration of benzoate, alone and with other substances. Since it is obviously impossible to discuss in detail all the vast amount of evidence which has accumulated, the following brief summary of the main investigations which bear upon the work of the present study is presented.

The results obtained by those who have used the first procedure have been varied, possibly because different species of experimental animals were used and because the dosage of benzoate varied widely. Wicchowski (9), McCollum and Hoagland (6), and Lewis (10, 11) reported that, after benzoate administration, the increase in the hippuric acid (glycine) nitrogen was accompanied by a corresponding decrease in the urea nitrogen and suggested that glycine was formed from some nitrogenous substance whose nitrogen was normally eliminated as urea. Magnus-Levy (3), Delprat and Whipple (12), and Ringer (4) concluded that the glycine resulted from a destruction of "extra" protein, above the amount normally catabolized, since they observed an increased elimination of total nitrogen. Epstein and Bookman (13) concluded that the production of hippuric acid resulted from a selective process which caused the elimination of large amounts of nitrogen, largely in the form of hippuric acid nitrogen.

The second type of experiment attacked the problem more directly, but had the disadvantage common to all perfusion experiments, the impossibility of exactly duplicating natural conditions of the living organism. Bunge and Schmiedeberg (14) noted a synthesis of hippuric acid after perfusion of the kidneys of dogs with glycine and sodium benzoate. Friedmann and Tachau (15) made similar observations, but employed rabbit livers for the perfusion. However, neither Friedmann and Tachau nor Hoffmann (16) were able to demonstrate that other amino-acids, hydroxy-acids, or fatty acids served as precursors of glycine in these experiments.

The third method has been most commonly used, but here also the results have been conflicting. Wiener (17) reported that in rabbits the subcutaneous injection of glycine, leucine, and sodium urate made toxic doses of benzoate non-toxic, but Cohn (18) was unable to confirm the results with leucine. Neither Abderhalden and collaborators (19) nor Delprat and Whipple (12) could establish the conclusion of Umber (20) that glycine was furnished by a process involving a change in the globulin-albumin ratio of the serum. Cohn (21) noted that the 24 hour output of hippuric acid in rabbits was increased when ammonium acetate was administered in addition to the benzoate, but he was unable to prove that this increase was not the result of greater protein catabolism due to the toxicity of the acetate. Ringer (4) observed that the administration of glycollic acid with benzoate to rabbits did not increase the hippuric acid formation, and Epstein and Bookman (22) obtained similar negative results with leucine and benzoate. Abderhalden and Strauss (5) found that in the pig the ingestion of glycine with benzoate increased the output of hippuric acid, but that neither alanine nor ammonium carbonate had any effect. The suggestion of Zimmermann (23) that glycine had its origin in the glycocholic acid of the bile was not confirmed by Kühne and Hallwachs (24), nor by Rosenberg (25), nor by Lewis (26).

Magnus-Levy (27) injected the benzoyl derivatives of alanine, valine, leucine, phenylalanine, ornithine, serine, aspartic acid, and glutamic acid subcutaneously into rabbits and found that all these substances were excreted in the urine unchanged. He concluded, therefore, that hippuric acid did not result from the formation and subsequent oxidation of these benzoyl compounds. Hippuric acid was isolated after the injection of the benzoyl derivative of an unidentified compound from the leucine fraction of a protein hydrolysate.¹

¹ The presence of hippuric acid after the administration of a benzoylated amino-acid is not necessarily proof of the oxidation of the compound to hippuric acid. It is possible that the benzoyl group might be split off either by the action of bacteria in the intestine or by enzymes in the body proper and that the benzoic acid thus formed would react with glycine as would ingested benzoic acid. Certain unpublished experiments by one of us (L.) indicate the possibility of such a reaction with dibenzoyl cystine.

It has been shown by Wiener (17) and also by Raiziss and Dubin (28) that the maximum synthesis of hippuric acid in rabbits occurred after the administration of 1 gm. of benzoic acid per kilo. The fatal dose for rabbits, however, has been found to be 1.7 gm. per kilo by Wiener (17) and from 1.5 to 2.4 gm. by Rost and coworkers (7). Ordinarily these animals can completely conjugate non-toxic doses of benzoate in 24 hours. The use of the 24 hour output of hippuric acid, after the ingestion of benzoate and a possible precursor of glycine, as an index of the availability of that substance as a source of glycine, as in most of the above experiments, necessitated the administration of doses of benzoate which could be completely synthesized into hippuric acid in 24 hours without the presence of extra glycine or of doses which were more or less toxic and often lethal. The question arises whether such results, particularly the negative results, really represent the ability of the organism to synthesize glycine.

It seemed probable that the formation of hippuric acid might proceed at a definite rate normally and that this rate might be increased by the presence of an excess of glycine or its precursors in the organism. If the rate of synthesis during a short period could be determined, this would give a method of experimentation which could be used after the ingestion of non-lethal doses of benzoate. In the following experiments a study has been made of the rate of synthesis of glycine in the 6 hour period after the administration of moderate amounts of benzoate, alone, and with possible precursors of glycine. The excretion of hippuric acid during this period was used as an index of the rate of formation of hippuric acid and, therefore, of glycine. Such a procedure, involving the use of non-toxic rather than toxic doses of benzoate, might give new and would certainly give more accurate information regarding the synthesis of glycine.

EXPERIMENTAL.

Normal adult rabbits were used in each series of experiments. The experimental period consisted of an experimental day with normal days immediately preceding and following. During each 3 day period the rabbits were fed milk, sufficient in quantity to maintain the body weight constant. This is a diet which is practically glycine-free. Between the experimental periods the

rabbits were given oats, carrots, and hay. On the experimental day the urine was collected 6 and 24 hours after the administration of the substances to be studied. The percentage of ingested benzoic acid which was excreted as combined benzoic during the 6 hour period was taken as a measure of the rate of synthesis of hippuric acid. The urines from the 2 normal days and the two urines from the experimental day were analyzed separately for free benzoic acid according to the method of Raiziss and Dubin (29), and for total benzoic acid according to the procedure of Kingsbury and Swanson (30). Combined benzoic acid or hippuric acid was found by subtracting the free benzoic acid from the total benzoic acid. It was assumed that all the combined benzoic acid was hippuric acid. After benzoate administration, benzoyl glycuronate is found in the urine of some types of animals; *e.g.*, the dog. By the method of Kingsbury and Swanson no distinction can be made between the different forms of combined benzoic acid. However, rabbits ordinarily excrete benzoyl glycine, benzoyl glycuronate being present in traces only, if at all. In all cases, the bladders of the rabbits were emptied by pressure at the beginning and at the end of each period. The urines were preserved with dilute nitric acid as suggested by Raiziss and Dubin (28).

In the tables, the figures for free and combined benzoic acid represent extra benzoic acid, a correction having been made for the normal excretion. Rabbits on a milk diet excrete daily from 0.008 to 0.012 gm. of free benzoic acid and from 0.060 to 0.100 gm. of combined benzoic acid. The former figures probably represent hydrolyzed hippuric acid since van de Velde and Stokvis (31) showed that if urine were collected directly from the ureters no free benzoic acid was present.

In order to make use of the excretion of hippuric acid during the 6 hour period as a measure of the rate of synthesis of glycine, it must be shown that the excretion of hippuric acid depends upon the amount of glycine available and not upon the rate of absorption of benzoate from the intestine, nor upon the rate of formation of hippuric acid, nor upon the rate of elimination of hippuric acid by the kidneys.

The excretion of hippuric acid after the oral administration of sodium benzoate was studied first. Rabbits were fed benzoate in

TABLE I.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate.

Rabbit No.	Weight.	Benzoic acid per kilo administered as sodium benzoate.	Extra benzoic acid excreted in 6 hr. period.		
			Free.	Combined.	
	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>
1	2.3	0.66		0.719	47
2	2.3	0.66	0.338	0.706	46
2	2.3	0.66	0.153	0.723	47
5	2.2	0.69	0.012	0.612	40
3	2.1	0.73	0.275	0.895	59
3	2.1	0.73	0.002	0.690	45
4	2.7	0.75	0.003	1.055	52
7	2.2	0.82	0.014	0.721	40
32	2.2	0.82	0.063	0.671	37
8a	1.8	0.85	0.081	0.881	58
6	2.1	0.86	0.019	0.866	48
30	2.1	0.86	0.038	0.721	40
47	2.3	0.87	0.074	0.903	45
41	2.2	0.89	0.030	0.670	34
44	2.6	0.89	0.258	0.957	42
31	1.8	0.90	0.098	0.651	40
46	2.2	0.91	0.078	0.815	41
43	2.5	0.92	0.040	0.592	26
33	2.3	0.94	0.070	0.757	35
33	2.3	0.94	0.064	0.716	33
9	1.9	0.95	0.070	0.630	35
23	1.5	0.96	0.026	0.538	37
12	1.8	1.00		0.770	43
45	1.9	1.00	0.094	0.605	32
28	1.9	1.04	0.020	0.828	42
29	1.9	1.05	0.065	0.597	30
35	1.4	1.11	0.015	0.738	51
8	1.6	1.12	0.009	0.635	35
27	1.6	1.12	0.008	0.694	38
27	1.6	1.12	0.008	0.699	39
Average of 30 experiments.....			0.072		41.2

doses of approximately 1 gm. of benzoic acid per kilo and the excretion of hippuric acid in the following 6 hour period was determined. The results are tabulated in Table I. In thirty

experiments, the average percentage of the benzoic acid ingested which was excreted as hippuric acid in the 6 hour period was 41.2 per cent. It is to be noted that these percentages are fairly constant, twenty-five being within a range of 30 to 50 per cent.

In order to be certain that these values represented the rate of synthesis and were neither an index of the rate of absorption of the benzoate from the intestine nor an index of the rate of excretion of hippuric acid by the kidney, these possibilities had to be eliminated. Proof that absorption from the intestine or elimination by the kidneys occurred faster than the hippuric acid synthesis would mean that these other processes could be disregarded as factors limiting the output of hippuric acid.

TABLE II.

Excretion of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Intravenous Administration of Sodium Hippurate.

Rabbit No.	Weight.	Benzoic acid per kilo administered as sodium hippurate.	Extra combined benzoic acid excreted in 6 hr. period.	
			gm.	per cent of intake
14	2.6	0.92	2.272	95
12	1.9	0.95	1.714	95
9	1.8	1.00	1.535	85
11	1.7	1.06	1.647	91
16	1.4	1.09	1.455	95

In the following experiments sodium hippurate, in amounts equivalent to about 1 gm. of benzoic acid per kilo, was injected into the ear veins of rabbits under urethane anesthesia and the excretion of hippuric acid in the 6 hour period determined. The solution injected contained 0.1 gm. of hippuric acid in 1 cc. of isotonic salt solution. The results are shown in Table II. It is evident that hippuric acid leaves the blood stream almost quantitatively during the 6 hour period, and that the rate of excretion by the kidney can be disregarded as a factor limiting the output of hippuric acid.

In order to rule out the factor of delayed absorption of sodium benzoate from the intestine, sodium benzoate was injected intravenously. Solutions of sodium benzoate containing 0.1 gm. of sodium benzoate in 1 cc. of isotonic salt solution were injected.

As in the previous experiments, the dosage used was approximately 1 gm. of benzoic acid per kilo. Table III shows the extent of hippuric acid excretion in the 6 hour period following the intravenous administration of sodium benzoate. In these experiments the rate of intestinal absorption could play no part, and since it has already been shown that there is no delay in the elimination of hippuric acid through the kidneys, these figures should repre-

TABLE III.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Intravenous Injection of Sodium Benzoate.

Rabbit No.	Weight.	Benzoic acid per kilo administered as sodium benzoate.	Extra benzoic acid excreted in 6 hr. period.		
			Free.	Combined.	
	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>
23	1.6	0.90	0.293	0.737	51
24	1.6	0.90	0.313	0.691	48
25	2.0	0.90	0.464	1.014	56
14	2.6	0.92	0.450	1.216	51
16	1.6	0.95	0.357	0.737	48
22	1.5	0.96	0.400	0.666	46
28	1.9	1.04	0.485	0.703	35
9	1.7	1.06	0.165	0.512	28
13	1.7	1.06	0.331	0.368	20
18	1.5	1.06	0.245	0.550	34
26	1.5	1.08	0.346	0.589	37
15	1.4	1.09	0.160	0.734	48
15	1.4	1.09	0.160	0.700	46
11	1.6	1.12	0.342	0.622	35
27	1.8	1.12	0.314	0.885	49
17	1.4	1.14	0.270	0.592	37
Average of 16 experiments.....			0.318		41.8

sent the actual rate of synthesis of hippuric acid. The average elimination (41.8 per cent) was almost the same as that found after enteral administration (41.2 per cent), showing that intestinal absorption of sodium benzoate was at least as rapid as the rate of synthesis.

It will be noticed in Tables I and III that, after benzoate administration, free benzoic acid was found in the urine. The

amount was especially large after intravenous administration, showing that temporarily the synthetic capacity of the tissues may be overwhelmed, which results in the escape of free benzoic acid into the urine.

6 hour urines were frequently found which reduced Benedict's solution, although the reduction was never heavy. In all cases in which reducing substances were present, the urine was tested with Fehling's solution, both hot, and in the cold for several hours. No reduction was obtained. Traces only, if any, of combined benzoic acid as glyceuronates were present.

Having determined the rate of synthesis of hippuric acid for the 6 hour period following the administration of benzoic acid in doses of about 1 gm. per kilo, the next step was to determine whether the administration of glycine simultaneously with the benzoate would have any effect on the hippuric acid output during this period. Nineteen rabbits were used for these experiments. For comparison, these experiments are tabulated (Table IV) in conjunction with the results obtained after the administration of benzoic acid alone. This makes it possible to observe the effect of the benzoate with and without the glycine on the same animal. Inspection of Table IV makes it evident that glycine had a marked effect in increasing the output of hippuric acid, almost doubling the quantity excreted in the 6 hour period in some experiments. Not only was the output of hippuric acid increased, but also in many cases the free benzoic acid excretion was decreased. This is very marked in Rabbit 2 (Table IV) where free benzoic elimination was decreased from 0.338 and 0.153 gm. to 0.004 and 0.003 gm. when the glycine was added. The results are not so striking in the experiments in which there was very little free benzoic acid after the administration of benzoate alone.

In the earlier experiments, five equivalents of glycine were administered in order to be sure that an excess was present. The result of decreasing the quantity of glycine is also shown in Table IV. In the experiments on Rabbit 27, three and one-half, two, and one equivalent of glycine all exerted a marked effect in increasing the 6 hour output of hippuric acid, two equivalents being more effective than three and one-half. Rabbit 31 showed a decided increase with three and one-half equivalents, and with one and one-half equivalents there was a noticeable effect. Rab-

TABLE IV.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone and Sodium Benzoate with Glycine.

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after ingestion of						Glycine.	Sodium benzo- ate.
	Benzoic acid.			Benzoic acid and glycine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>		
2*				0.004	0.964	63	5	1.8
	0.338	0.706	46					1.8
	0.153	0.723	47					1.8
3				0.003	1.051	69	5	1.8
	0.275	0.895	59					1.8
	0.002	0.690	45	0.003	0.999	66	5	1.8
				0.002	1.065	70	5	1.8
4	0.003	1.055	52					2.4
				0.003	1.571	77	5	2.4
5				0.005	1.030	68	5	1.8
	0.012	0.612	40					1.8
6	0.019	0.866	48					2.12
				0.013	1.328	74	5	2.12
7	0.014	0.721	40					2.12
				0.009	1.270	71	5	2.12
8				0.010	1.215	80	5	1.8
23				0.115	0.895	62	5	1.7
	0.026	0.538	37					1.7
				0.069	0.592	41	2	1.7
				0.024	0.672	55	3	1.7
27	0.008	0.694	38					2.12
				0.018	1.408	78	3½	2.12
				0.006	1.456	81	2	2.12
	0.008	0.699	39					2.12
				0.025	1.121	62	1	2.12

* The weight of the rabbits used in these and subsequent experiments is given in Tables I, II, and III.

TABLE IV—*Concluded.*

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after ingestion of						Glycine.	Sodium benzo- ate.
	Benzoic acid.			Benzoic acid and glycine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>mol</i>	<i>gm.</i>
28	0.020	0.828	42	0.004	1.334	67	5	2.34 2.34
30	0.038	0.721	40	0.076	1.369	76	2	2.12 2.12
31	0.098	0.651	40	0.005	1.308	81	3½	1.91 1.91
				0.100	0.907	56	1½	1.91
32	0.063	0.671	37	0.008	0.717	40	2	2.12 2.12
				0.007	1.303	73	3	2.12
33	0.070	0.757	35	0.054	1.516	70	3½	2.55 2.55
				0.035	1.630	75	2	2.55 2.55
	0.064	0.716	33					
35	0.015	0.738	51	0.027	1.219	84	3	1.7 1.7
43	0.040	0.592	26	0.024	0.721	31	2	2.72 2.72
44	0.258	0.957	42	0.001	1.824	79	2	2.72 2.72
46	0.078	0.815	41	0.005	1.513	76	2	2.36 2.36
47	0.074	0.903	45	0.078	0.803	40	2	2.36 2.36
Average..	0.076		41.9	0.026		66.6		
Average of experiments with three or more equivalents of glycine.....						71.0		

bit 33 showed a striking effect with both three and one-half and two equivalents. However, this increase was not observed in all of the animals after the administration of the smaller quantities of glycine with benzoate. With Rabbit 32, three equivalents were effective, but there was no increase after two equivalents. It is interesting to compare the results obtained after benzoate alone and after benzoate with glycine (Table IV). In the former experiments the average excretion of hippuric acid for the 6 hour period was 42 per cent, while after the ingestion of both benzoate and glycine the same rabbits excreted 67 per cent of the ingested benzoate as hippuric acid. If those experiments are omitted in which less than three equivalents of glycine were used, the average was 71 per cent. For these same rabbits, the average excretion of free benzoic acid after benzoate feeding was 0.076 gm., and after benzoate and glycine, 0.026 gm.

The increased output of hippuric acid observed when glycine was administered with the benzoate might be due to: (a) an increased rate of absorption of benzoic acid from the intestine, or (b) a stimulation of general metabolism, or (c) an increased rate of synthesis of hippuric acid due to the presence of an excess of glycine in the organism.

If the formation of hippuric acid is delayed by the slow absorption of benzoic acid from the intestine, then glycine might increase the hippuric acid output by increasing the rate of absorption. That this was not the case was shown by the following experiments (Table V) in which the benzoic acid was administered intravenously, and the glycine given either *per os* or subcutaneously. Glycine produced the same effect on the excretion of hippuric acid whether benzoic acid was administered *per os* or intravenously. Therefore, this effect was not due to increased absorption from the intestine. It is also evident that there was no delay in the absorption of the benzoate from the intestine.

Lusk (32) has shown that certain amino-acids exert a striking effect on metabolism, causing an increased respiratory exchange and an increased heat production, a phenomenon known as specific dynamic action. He showed that the amino-acids, glycine and alanine, were particularly active in stimulating metabolism in this way. If the increased output of hippuric acid after benzoate and glycine administration were due to the general stimulation of

metabolism by glycine, the same increase should follow the ingestion of benzoate and alanine. However, this was not found to be the case. In Table VI are given the results of experiments in which alanine was fed with benzoate. In eight experiments there was no evidence of increased hippuric acid excretion except in Rabbit 5. In view of the other results, this apparent effect

TABLE V.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Intravenous Administration of Sodium Benzoate Alone and Sodium Benzoate plus Glycine. (Glycine Administered per Os, or Subcutaneously.)

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after administration of						Glycine.	Sodium benzoate
	Benzoic acid.			Benzoic acid and glycine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>mol</i>	<i>gm.</i>
14	0.450	1.216	51	0.284	1.700	71	5†	2.83 2.83
15	0.160	0.734	48	0.245	0.874	57	5†	1.8 1.8
16	0.357	0.737	48	0.251	1.012	66	5‡	1.8 1.8
47	0.074	0.903	45*	0.318	1.258	63	3†	2.36 2.36

* Benzoate per os.

† Per os.

‡ Subcutaneously.

was probably due to an individual variation in this rabbit. Almost the same variation is seen in Table I for Rabbit 3, in which the combined benzoic acid elimination after benzoate ingestion was 59 per cent on one occasion and 45 per cent in a later experiment. In the alanine feeding experiments there was no decrease in the amount of free benzoic acid eliminated. The average combined benzoic acid excretion for these eight rabbits was 42

per cent after the administration of alanine with benzoate and 44 per cent after benzoate alone. It would appear that stimula-

TABLE VI.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone and Sodium Benzoate plus Alanine.

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after administration of						Alanine.	Sodium benzo- ate.
	Benzoic acid.			Benzoic acid and alanine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>mol</i>	<i>gm.</i>
2	0.153	0.723	47	0.044	0.707	46	5	1.8 1.8
4	0.003	1.055	52	0.114	0.892	46	5	2.4 2.4
5				0.055	0.876	57	5	1.8 1.8
	0.012	0.612	40					
6	0.019	0.866	48					2.12 2.12
				0.080	0.857	48	5	
7	0.014	0.721	40					2.12 2.12
				0.031	0.639	35	5	
15	0.100	0.700	46*					1.8 1.8
				0.226	0.572	38*	5	
30	0.038	0.721	40					2.12 2.12
				0.083	0.596	33	3	
31	0.098	0.651	40					1.91 1.91
				0.097	0.605	37	3	
Average..	0.062		44.1	0.085		42.5		

* Benzoate injected intravenously.

tion of the general metabolism by amino-acids cannot explain the increased hippuric acid synthesis observed after glycine administration.

It seems quite probable that the increased excretion of hippuric acid after the administration of glycine and sodium benzoate resulted from an increase in the rate of synthesis of hippuric acid due to the abundant amounts of free glycine present in the organism. *It is evident that with a constant ingestion of benzoate, the quantity of hippuric acid which can be synthesized in a definite period of time is not limited by a fixed rate of conjugation of benzoic acid with glycine, but is dependent upon the amount of glycine available in the organism.* Since the rate of synthesis of hippuric acid does depend upon the amount of glycine available and since ingested glycine does increase the rate of synthesis of hippuric acid, it should be possible to use this procedure in determining whether certain other substances are precursors of glycine. It has been shown that alanine is not such a precursor since there was no increase in the hippuric acid excretion following its administration with sodium benzoate (Table VI).

It was thought that nor-leucine and leucine might differ in their effects when administered with benzoate. Magnus-Levy (27) found that benzoyl leucine was not broken down to give hippuric acid. He did not study benzoyl nor-leucine. Benzoyl nor-leucine might be formed and oxidized to benzoyl glycine. Some difficulty was found in feeding these amino-acids, nor-leucine and leucine, because of their insolubility. They dissolve readily in acid or alkali, but the resulting solutions are strongly acidic or alkaline. The procedure finally adopted was to dissolve half the amino-acid in dilute hydrochloric acid and half in an equivalent amount of dilute sodium hydroxide. The two solutions were then administered alternately in small portions through a stomach tube.

The results after the administration of nor-leucine and leucine are given in Tables VII and VIII, respectively. In these experiments the amino-acids were given *per os* and the benzoate was also given *per os* unless otherwise indicated in the tables. Examination of these tables shows that neither of these two amino-acids caused an increased rate of elimination of hippuric acid, nor was the elimination of free benzoic acid decreased. Rabbit 6 (Table VII) appeared to show an increase from 48 to 66 per cent. This result is questionable, however, because the urine was contaminated with feces, and the recovery of extra benzoic acid in the

24 hour period amounted to 129 per cent, indicating the probable presence of titratable substances other than benzoic acid. In some of these experiments more or less toxic symptoms resulted,

TABLE VII.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone and Sodium Benzoate plus Nor-Leucine.

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after administration of						Nor-leucine.	Sodium benzoate.
	Benzoic acid.			Benzoic acid and nor-leucine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>mol</i>	<i>gm.</i>
2				0.240	0.460	26	2	2.12
				0.500	0.585	32	3½	2.12
6	0.019	0.866	48					2.12
				0.556	1.196	66*	5	2.12
8a				0.049	1.036	68	6	1.8
	0.021	0.973	64					1.8
17	0.270	0.592	37†					1.89
				0.180	0.710	44†	4	1.89
23	0.293	0.737	51†					1.7
				0.220	0.568	39†	5	1.7
28				0.047	0.960	48	5	2.34
	0.020	0.828	42					2.34
31	0.098	0.651	40					1.91
				0.068	0.586	36	3	1.91
39	Fatal in 4 hrs.						6	1.8

* Recovery of benzoic acid in 24 hour period was 129 per cent. Urine was contaminated with fecal material.

† Benzoate injected intravenously.

especially the occurrence of diarrhea. This toxicity was particularly noticeable after the leucine administration, three of the experiments being fatal.

In order to determine the effect of other amino-acids, isovaline, aspartic acid, and cystine were administered orally with sodium benzoate. Glycollic acid and glycol aldehyde were also administered with benzoate to see whether these substances were precursors of glycine. The aspartic and glycollic acids were neutralized before administration. None of these substances increased the rate of synthesis of hippuric acid (Table IX). The result after the administration of glycol aldehyde may be question-

TABLE VIII.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone and Sodium Benzoate plus Leucine.

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after administration of						Leucine.	Sodium benzoate.
	Benzoic acid.			Benzoic acid and leucine.				
	Free.	Combined.		Free.	Combined.			
	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent of intake</i>		
7	0.014	0.721	40	0.310	0.523	29	3½	2.12
23	0.293	0.737	51*	0.265	0.683	47*	5	1.7
24	0.313	0.691	48*	0.260	0.418	29*	5	1.7
	(Rabbit died following day.)							
22	Fatal in 8 hrs.*						5	1.7
33	Fatal in 4 hrs.*						2½	2.55

* Benzoate injected intravenously.

able in view of the fact that one experiment was terminated fatally in 6½ hours; *i.e.*, the animal was subnormal the latter part of the 6 hour period.

Glucose might also serve as a source of glycine by cleavage to acetic acid or acetaldehyde, or some other intermediary metabolite. Urea might increase the rate of synthesis of hippuric acid either by conversion to glycine or by increasing the amount of ammonia in the organism according to the mass law through the

TABLE IX.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone and Sodium Benzoate plus Each of the Following: Isovaline, Aspartic Acid, Cystine, Glycollic Acid, and Glycol Aldehyde.

Rabbit No.	Extra benzoic acid excreted in 6 hr. period after administration of						Substance administered.	Sodium benzoate.
	Benzoic acid.			Benzoic acid and experimental substance.				
	Free.	Combined.		Free.	Combined.			
	gm.	gm.	per cent of intake	gm.	gm.	per cent of intake	mol	gm.
27	0.314	0.885	49*	0.297	0.662	37*	Isovaline.....	5 2.12
				0.129	0.490	30	Aspartic acid.....	5 2.12
	0.008	0.699	39					2.12
26	0.346	0.589	37*	0.272	0.382	24*	Aspartic acid.....	5 1.91
				Fatal in 10 hrs.				1.91
8a	0.081	0.881	58	0.055	0.537	35	Cystine.....	5 1.8
				Rabbit died on following day.				1.8
40	0.028	0.852	47	0.215	0.655	36	Glycollic acid.....	3½ 2.12
32	0.063	0.671	37	0.058	0.535	30	Glycollic acid.....	5 2.12
44	0.258	0.957	42	0.011	0.587	26	Glycol aldehyde†....	3 2.71
				Fatal in 6½ hrs.				2.71
52‡	0.070	0.626	31	0.010	0.552	27	Glycol aldehyde....	3 2.36
				Rabbit died on following day.				2.36

* Benzoate injected intravenously.

† Acknowledgement is made to Dr. Isidor Greenwald of the Harriman Research Laboratory, Roosevelt Hospital, New York, for the glycol aldehyde used in the experiments.

‡ After the administration of 2.36 gm. of sodium benzoate and three equivalents of glycine this rabbit eliminated 67 per cent of the ingested benzoic acid as hippuric acid in the 6 hour period immediately following ingestion of the benzoate.

slowing up of the reaction, ammonia \rightarrow urea. If acetic acid is a precursor of glycine, then the ingestion of the acetate group should increase the rate of synthesis of hippuric acid. The results following the administration of these substances with benzoate are given in Table X. Glucose, urea, and sodium acetate were not found to increase the rate of synthesis of hippuric acid. Ammonium acetate and the larger doses of urea were rapidly fatal.

TABLE X.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate with Each of the Following: Glucose, Urea, and Sodium Acetate.

Rabbit No.	Weight	Extra benzoic acid excreted in 6 hr. period.			Substance administered.
		Free.	Combined.		
	kg.	gm.	gm.	per cent of intake	
30	2.1	0.029	0.693	38	1.8 gm. benzoic acid as Na salt and 20 gm. glucose <i>per os</i> .
48	2.8	0.026	1.100	44	2.5 gm. benzoic acid as Na salt and 10 gm. glucose and 0.8 gm. urea <i>per os</i> .
35	1.4	0.008	0.684	47	1.44 gm. benzoic acid as Na salt and 1.5 gm. urea <i>per os</i> .
49	2.8	0.293	0.677	27	2.5 gm. benzoic acid as Na salt, 5.0 gm. Na acetate, and 0.8 gm. urea <i>per os</i> .
50	2.8	0.090	0.957	38	2.5 gm. benzoic acid as Na salt and 5.0 gm. Na acetate <i>per os</i> .

DISCUSSION.

Of all the compounds studied glycine was the only substance which increased the rate of synthesis of hippuric acid after benzoate ingestion. The other amino-acids and related substances which were tested gave negative results. The results were clear-cut and striking, practically without exception. Three

equivalents or more of glycine with benzoate almost doubled the rate of synthesis of hippuric acid, while the other substances with benzoate gave results similar to and, for the most part, slightly lower than the results with the benzoate alone.

In the synthesis of glycine it is assumed that one or more intermediate products are formed. The administration of one of these intermediate products, or of a compound which breaks down to give one of these products, ought to increase the rate of synthesis of glycine and, therefore, of hippuric acid. It would appear that none of these amino-acids or related substances gave rise to precursors of glycine in the organism. It is reasonable to suppose that the deamination and oxidation of alanine, nor-leucine, leucine, cystine, isovaline, and aspartic acid are typical of amino-acids in general. Since these substances were not shown to be precursors of glycine, it may be concluded that typical oxidative deamination does not give rise to precursors of glycine. It does not necessarily follow that no other amino-acid may act as a precursor of glycine. It is probable, however, that if any furnish glycine, they do so by some special catabolism and not by catabolism in the usual manner. Benzoyl derivatives of other amino-acids than those which were studied by Magnus-Levy (27) may be formed and oxidized to hippuric acid. Knoop (33) has suggested a possible formation of glycine from α -amino- β -hydroxy-acids, such as serine, or hydroxyglutamic acid.

In view of the structural relationship between glycine and acetic, glycollic, and glyoxylic acids, these latter substances have been considered as intermediate compounds in the metabolism of glycine by the organism. Neubauer (34) suggested that glycine gave rise to glycollic and glyoxylic acids on the basis of his experiments in which phenylglycollic and phenylglyoxylic acids were found in the urine after the administration of phenylglycine to dogs. He postulated that amino-acids in general gave hydroxy- and keto-acids by oxidative deamination. The reverse of this reaction, the formation of amino-acids from hydroxy- and keto-acids, was noted by Knoop (35) and his collaborators. Embden and Schmitz (36) showed that alanine was formed from pyruvic acid and from ammonium lactate by perfusion of the liver. However, the conversion of the glycollic and glyoxylic acids to glycine has not been observed. Haas (37) could find no evidence for the

formation of glycine from ammonia and glyoxylic acid in the rabbit, and Sassa (38) was unable to demonstrate a production of glycine from glycollic acid in the same species. Thus, in spite of structural similarity this conversion of glycollic and glyoxylic acids to glycine by the organism has not yet been demonstrated.

The failure of urea, glucose, and sodium acetate to act as precursors of glycine is consistent with the negative results obtained after the administration of the amino-acids, alanine, leucine, nor-leucine, etc., since amino-acids themselves are catabolized to urea and to fatty acids which give acetic acid by oxidation, and glucose is assumed to give acetic acid or acetaldehyde as an intermediate stage in its oxidation. These results are concordant since it is reasonable to expect that if the amino-acids and glucose give negative results, urea and sodium acetate would have the same effect.

In drawing conclusions from experiments of this nature, it must be borne in mind that negative results do not have the significance of positive results. There is to be considered, for instance, the undetermined influence of the time element. The 6 hour period may have been too short for synthesis of glycine to have been effected from these amino-acids and related substances. This would, however, involve the assumption that metabolic, or at least synthetic, processes take place at a slower rate than is commonly believed. Csonka (39) showed that in the phlorhizinized dog, the maximum conversion of glycine and alanine to urea and glucose took place in the 2nd hour after the administration of these amino-acids. On the other hand, Folin and Berglund (40) found that in men the maximum concentration of urea in the blood and urine occurred 6 to 8 hours after the ingestion of gelatin, although amino-acids in the blood reached a maximum in the 2nd hour. Such findings relative to the velocity of body processes are suggestive of the unknown factors that may have a bearing on this problem of glycine synthesis.

A summary of the experimental results following the administration of hippuric acid, benzoic acid, and benzoic acid with amino-acids and related compounds is presented in Chart 1. These results, which have been previously discussed, show very clearly the difference between glycine and the other substances in their effect on the excretion of hippuric acid.

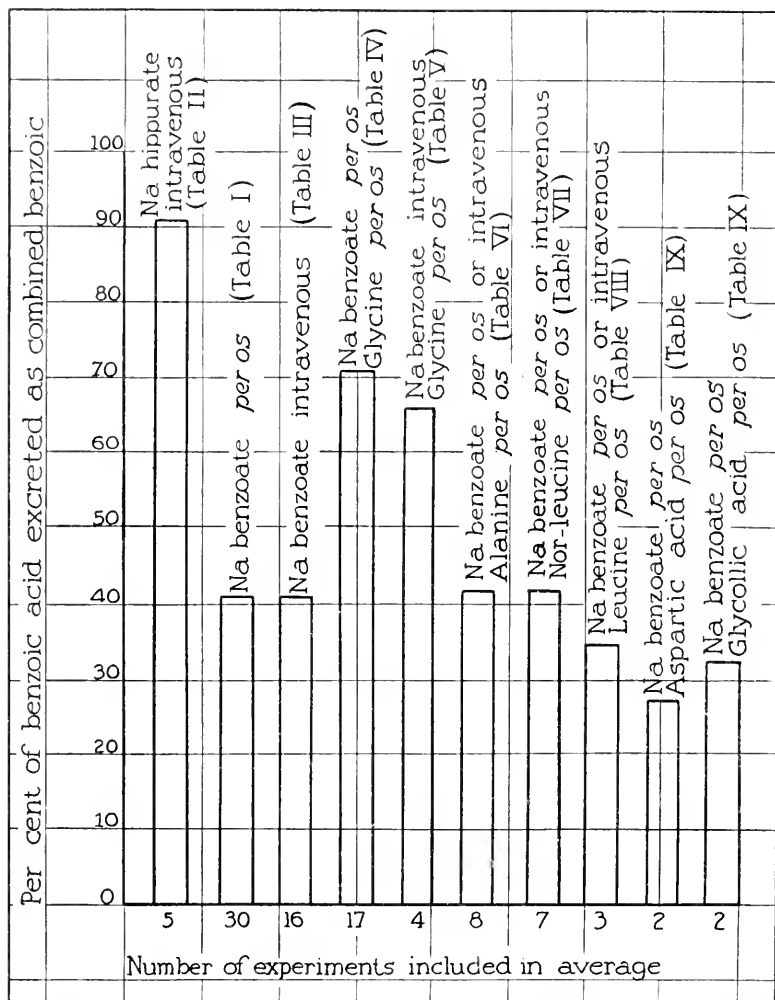


CHART 1. The average excretion of extra combined benzoic acid in the 6 hour period following the administration of hippuric acid, benzoic acid, and benzoic acid with amino-acids and related substances. The hippuric and benzoic acids were administered in doses of approximately 1 gm. of benzoic acid per kilo.

SUMMARY.

1. A study has been made of the rate of excretion of hippuric acid in rabbits during a 6 hour period after the oral and intravenous administration of sodium benzoate in doses of approximately 1 gm. of benzoic acid per kilo of body weight.

2. The rate of excretion of hippuric acid was decidedly increased by the administration of glycine with sodium benzoate.²

3. This increase was not the result of a stimulation of the excretory function of the kidneys by glycine since sodium hippurate alone, in amounts equivalent to the benzoate ingested, was completely excreted in the 6 hour period following its intravenous administration. The increase was not due to a faster rate of absorption of benzoate from the intestine because of stimulation of absorption by glycine since the same increase was observed when benzoate was administered intravenously and glycine orally or subcutaneously. The increase was not caused by stimulation of general metabolism, since alanine, which also stimulates general metabolism, was not found to increase the rate of excretion of hippuric acid.

4. The increased rate of excretion after the ingestion of glycine with benzoate probably represented an increased rate of synthesis of hippuric acid due to the presence in the organism of large amounts of preformed glycine.

5. Other amino-acids, alanine, cystine, leucine, nor-leucine, isovaline, and aspartic acid, did not increase the rate of synthesis of hippuric acid when administered with the benzoate. Similar negative results were obtained with glycollic acid, glycol aldehyde, glucose, urea, and sodium acetate. Hence it was considered improbable that any of these substances is a readily available precursor of glycine.

² Since the publication of our preliminary report of this work (Lewis, H. B., and Griffith, W. H., *J. Biol. Chem.*, 1923, lv, p. xxii), Kingsbury (Kingsbury, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 405) has reported that in two normal men with a low rate of elimination of hippuric acid after benzoate ingestion, the rate of hippuric acid excretion was notably accelerated by the ingestion of glycine in amounts equivalent to the benzoate fed.

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THE INFLUENCE OF THE POSITIVE NITROGEN BALANCE UPON CREATINURIA DURING GROWTH.*

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As a result of a study of the excretion of creatine in children when on high protein diets (1), the authors had come to believe that there existed a definite maximum production of creatine. The total creatine produced included the creatine excreted plus the creatinine (in terms of creatine). The work of Shaffer (2), of Myers and Fine (3), and of Rose, Dimmitt, and Cheatham (4), furnished, in our opinion, sufficient justification for such a procedure. It was found that the sum of the two formed a constant production per kilo of body weight, independent of the age of the child. As it happened that the total creatine coefficient of children was of the same magnitude as the creatinine coefficient of adult man, this furnished an explanation of the failure to produce creatinuria in adult man by high protein feeding, and offered, at any rate, a partial explanation of the irregular results found in the creatinuria of women. An extension of the idea showed also that the creatinuria of some pathological conditions, and their dependence upon dietary protein, could be considered from the same standpoint.

The excretion and formation of creatinine in children is a constant quantity during the relatively short experimental period. The creatine excretion is thus the only variant with the dietary protein, and the assumption that the production of new or excess creatine reaches a maximum beyond which no amount of further protein feeding can increase, is not in harmony with many of the views which have more recently been put forward on the origin of

* The facts of this and the preceding paper (1) by the same authors, are taken from a thesis submitted as a partial requirement for the degree of Doctor of Philosophy in the University of Toronto.

that substance (5). Consequently, it becomes necessary to inquire more particularly into the possibility of a creatine maximum. The diets which we gave our children, though high in protein, could not be considered as excessively so, except perhaps in the case of the youngest group. They were, however, as high as the children could conveniently consume. Experimental animals offer a much better opportunity for study in this respect, and we have a number of observations on growing dogs, which fulfill all the requisite experimental conditions. The puppy (6), like the child, is continually excreting creatine as a physiological occurrence, and the amount is in most cases, dependent upon the protein intake (7). In carrying out creatine metabolism experiments, it is of the utmost importance that the animals are in normal condition. We have emphasized this in our experiments upon children, but it is even of more importance in animal experimentation in this subject. Not all laboratory animals are in such condition, even when they appear active and vigorous. Their growth is often spasmodic, and especially do they show irregularities in their creatinine output and coefficients. This is particularly noticeable after a period of fasting, or of negative nitrogen balance and such effects can be observed for even so long as a month after the cause. Only three of our animals fulfil such conditions, and all of them had been objects of care, and properly nourished before their entrance into the laboratory. In working with small animals of this kind, it must be borne in mind that the rapid growth changes which take place, with the formation of new tissue, and especially muscle, must lead to the production of much new creatine which never finds its way into the urine. This fraction of the creatine production, absorbed or retained by the new muscle, may be greater in amount than the creatine excreted. From the data presented in this paper, its amount can be calculated, and we have assumed that creatine absorption (including that required for creatinine) + creatine excretion = creatine production. This, though undoubtedly true in a general way, will not hold in every individual case, as the calculation does not take into account any fluctuations in the percentage of creatine in muscle, or the possibility of variation in excretion due to changes in the blood, or in renal permeability. Such changes we hope we have avoided by the rigid selection of animals and experimental conditions.

The experiments reported are upon three female puppies. All of them were of the terrier type, two of them, (Dogs D and K) being fox-terriers of good pedigree, and from the same litter, while the third, (Dog L. B.) possessed a strain of bull-terrier. All the animals had been for many weeks previous to these experiments, upon a creatine-free diet, and had been living in metabolism cages. These experiments had their origin in an attempt to find conditions in puppies, when the creatine excretion would be unaffected by growth changes. Such conditions we expected to find on a pro-

TABLE I.

Dog.	Diet.	Length of experiment.	Average weight.	Daily N intake.	Per kg. body weight per day.				Average daily creatine.
					Total N intake.	N of urine.	N balance.	Creatinine.	
		<i>days</i>	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mj.</i>	<i>mj.</i>
D*	1	12	2.35	1.33	0.56	0.33	0.14	21.3	56
	2 and 3.	14	2.71	2.66	0.98	0.48	0.34	22.9	88
	4	11	3.30	5.33	1.61	1.00	0.37	23.3	89
K†	1*	15	2.49	1.33	0.53	0.32	0.06	19.3	77
	2* and 2.	13	2.61	2.66	1.01	0.54	0.33	20.6	62
	4	14	2.35	5.33	1.58	0.97	0.38	24.2	64
L.B.‡	5	5	9.15	2.00	0.22	0.19	0.00	27.9	108
	6	8	9.39	5.55	0.59	0.26	0.24	27.6	80
	7	4	9.92	10.88	1.10	0.29	0.29	27.7	76

* Protocol 1.

† Protocol 2.

‡ Protocol 3.

tein diet, complete in amino-acids, and of such a height that the rate of growth had reached a maximum. Any further additions of protein, or of individual amino-acids should then reflect themselves quantitatively in creatine excretion, were creatine an exogenous product derived from protein. The experiments, consequently, are a record of the creatine excretion of puppies on a series of protein diets of increasing height. As in each animal we have a series of three such diets, we have designated them as low, intermediate, and high protein diets, respectively, for purposes of discussion. The results are collected together in Table I. The diets

TABLE II.

Diets.

Diet No.	1	2	3	4	5	6	7	8	9
Bread	35.0 gm.	35.0 gm.	35.0 gm.	35.0 gm.	80.0 gm.			35.0 gm.	70.0 gm.
Corn-starch ..	30.0 "	30.0 "	60.0 "	30.0 "	45.0 "	100.0 gm.	100.0 gm.	30.0 "	30.0 "
Butter	12.0 "	6.0 "	6.0 "	6.0 "	35.0 "			6.0 "	12.0 "
Meat powder ..	2.0 "	8.5 "	8.5 "	8.5 "				20.0 "	11.0 "
Casein				25.0 "			50.0 "		
Milk	100.0 cc.	200.0 cc.	200.0 cc.	200.0 cc.	125.0 cc.	1,100.0 cc.	1,100.0 cc.	200.0 cc.	300.0 cc.
Cod liver oil, malt	15.0 "	15.0 "	15.0 "	15.0 "				15.0 "	15.0 "
Carbohydrate ..	52.0 gm.	56.0 gm.	81.0 gm.	56.0 gm.	85.0 gm.	129.0 gm.	129.0 gm.	56.0 gm.	77.0 gm.
Fat	22.0 "	21.0 "	21.0 "	21.0 "	35.0 "	44.0 "	44.0 "	21.0 "	29.0 "
Protein	8.3 "	16.7 "	16.7 "	32.5 "	11.0 "	35.0 "	67.0 "	27.0 "	25.4 "
Nitrogen	1.33 "	2.66 "	2.66 "	5.33 "	2.0 "	5.55 "	10.88 "	4.30 "	4.10 "
Calories	451.06	492.00	595.00	545.00	718.0	1,080.00	1,210.00	535.00	700.00

Diets 1*, 2*, and 9* are the same as diets 1, 2, and 9, without cod liver oil and malt extract.
5.6 gm. of sodium acetate were added to diet 5, and sodium bicarbonate to diet 7.

are given in Table II. The figures upon which the averages and calculations are based, are to be found in protocols at the end of this paper. The N intake, urinary N, and N balance are expressed in average grams per kilo of body weight per day. As far as we know the diets were complete in amino-acids. The N content of the diets was determined by previous analyses of the various constituents. These analyses were checked from time to time, and found to vary only slightly. The N balances were determined experimentally in Dogs D and K, and calculated on Dog L. B., on a basis of 85 per cent absorption. This absorption coefficient was found to be very constant for all the other puppies examined in the laboratory, and it is safe to assume its constancy in Dog L. B.

The Kjeldahl-Gunning method was used for the determination of total N, while the micro method of Folin was used for the determination of creatine and creatinine. Fecal N was determined by emulsifying the feces with 20 per cent sulfuric acid, making up to a definite volume, and determining the N on an aliquot portion by the Kjeldahl method.

An inspection of Table I shows that in each animal the increases in the levels of protein intake represent increases per kilo of body weight. The ratios of these changes are

Dog.	Ratio of protein intake.
D.....	1:1.75:3
K.....	1:1.9 :3
L. B.....	1:2.7 :5

The catabolic N varies somewhat similarly.

Dog.	Ratio of catabolic N.
D.....	1:1.45:3.3
K.....	1:1.4 :3
L. B.....	1:1.37:3.4

The N balances, however, show that a maximum rate of N retention is almost reached on the intermediate diet; it is only very slightly increased on the high diet. The conditions then, in these three animals, should be almost ideal for observing the effect of

increase of protein intake, or of catabolized protein, upon creatine excretion. With no further rate of demand for creatine to supply new tissue, the creatine excretion should then become a measure of creatine production, were this latter entirely dependent upon either of those factors.

Creatine excretion, however, becomes constant. Per kilo of body weight, creatine excretion declines slightly. As at the same time, creatine absorption by the new tissue becomes approximately constant, or only increases very slightly (judged by the N

TABLE III.

Dog.	Diet.	Length of experiment.	N intake.	Per kg. body weight per day.				Average daily creatine.	Total creatine coefficient.
				N intake.	Urine N.	N balance.	Creatinine.		
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
D*	1*	19	1.33	0.565	0.37	0.07	20.0	93	66.7
	†	12	1.33	0.565	0.33	0.14	21.2	56	55.1
	8	10	4.30	1.15	0.67	0.31	24.7	22	52.5
	8	11	4.30	1.08	0.82	0.08	27.1	85	52.8
	8	10	4.30	1.04	0.77	0.04	28.0	119	62.4
K†	2* and 2.	13	2.66	1.00	0.53	0.33	20.5	62	65.1
	2 and 3.	14	2.66	0.91	0.52	0.23	22.0	87	67.6
§	8	8	4.30	1.23	0.80	0.22	23.9	31	46.5
	8	15	4.30	1.09	0.65	0.26	23.7	14	43.4
	8	8	4.30	1.03	0.70	0.12	25.6	45	44.0

* Protocol 1.

† Protocol 4.

‡ Protocol 2.

§ Protocol 5.

balances), it is evident that the production of creatine becomes constant. In view of the evident independence of creatine excretion and catabolic N under these conditions, and its dependence upon N balance rather than N intake, it becomes necessary to observe more closely these variants, and their effect upon creatinuria. This effect is shown in Table III on Dogs D and K. It will be seen that comparisons are made between the creatine excretions of different experimental periods with constant N intake. The experimental records on each dog for each level of

total protein intake are consecutive. In Dog D on diets 1* and 1, Dog K on diets 2* and 2, and diets 2 and 3, and again in Dogs D and K on the two latter periods of diet 8, the N intake and urine N per kilo of body weight, undergo no alteration. The N gain undergoes marked fluctuation, with an inverse fluctuation in creatine excretion. In Dog D on diet 8, we have a decreasing N intake per kilo of body weight, especially marked in the first portion of the period, with a catabolic N per kilo of body weight, showing a maximum in the central portion of the experimental period. The N gain shows a marked fall with each period, and the creatine excretion, an equally marked rise. It is interesting to note, that the alteration in the N balance in Dog D on diet 1, was brought about by the addition of a small amount of cod liver oil and malt extract. Presumably, the animal was in a condition of vitamin shortage in spite of the milk and butter in the diet. Some of our other animals, especially the younger ones, showed similar changes.

This interrelationship of the positive nitrogen balance and creatinuria, may explain a number of results in the literature, in which daily variations in creatine excretion are apparently connected with catabolic N. Such a connection will become apparent in a subject at approximately constant weight, on a constant diet, or where alterations in the N of the diet are of such a character as to change simultaneously both the N balance and catabolic N, but in opposite directions.

The relationship of N balance and creatine excretion, though still evident, very often becomes disturbed in animals which are not strictly normal. An evident illustration of this was observed in animal T. B. The animal was distinctly not a normal animal. She only grew at about one-third the normal rate, would have occasional days of diarrhea, fine tremors constantly passed over the body, and occasionally larger convulsive movements of the head and neck were observed. The observations in Table IV were made after a short period of fasting, and when the animal was clinically greatly improved. In the first three periods, the creatine excretion runs parallel with the catabolic N, and inversely with the N gain. In the fourth period, however, there is no alteration in the N intake, gain, or balance, and a sudden drop in the creatine excretion. This coupled with a rapid rise in the creatinine excretion, would incline one to believe that there had been a sudden

elaboration of muscle, with a consequent demand for creatine. Though this case is undoubtedly an exaggerated one, it is certain that similar changes in the creatine content of the body occur in other animals, and it is only by a rigid selection of those animals, which show freedom from spasmodic growth upon regular diets, that some of the irregularities in creatine excretion can be minimized.

The calculation of the amount of creatine absorbed by the new growth is dependent upon several factors, some of which are variable in the dog, owing to the wide variations in breeds of this animal. For the relationships of N balance, growth, and musculature, we have used the data of our own experiments and some data of Falck (8). The creatine content of dog muscle is taken as 0.37 per cent as the average figure, from the estimations of Myers

TABLE IV.

Dog.	Diet.	Length of experiment.	N intake.	Per kg. body weight per day.				Average daily creatine.
				N intake.	Urine N.	N balance.	Creatinine.	
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>
T. B.	9 ^x	5	4.10	0.74	0.37	0.23	27.7	2
	9 ^x	5	4.10	0.71	0.51	0.02	29.5	21
	9 ^x and 9.	7	4.10	0.71	0.57	0.00	29.5	69
	9	10	4.10	0.71	0.58	0.00	31.3	9

and Fine (3), and Baumann and Hines (9). The use of the figure 0.37 per cent for the concentration of creatine in muscles of our puppies appears justifiable in the light of the findings of Mellanby (10) on growing chicks and rabbits, and of Myers and Fine (3) on growing cats. In all cases, the creatine content of the muscle reaches an adult figure at a very early stage in growth. An inspection of the creatinine coefficients of our animals (Table VIII) will show that they had almost reached maturity by the end of our experiments, and we only commenced our experiments when the animals were from one-third to one-half grown. Falck supplies the only data of the effect of growth upon the relationship of the body weight and musculature in the dog. The data of Voit (11) on the musculature of dogs of different weight, leave one in doubt as to whether the differences are to be ascribed to age or

breed. Falck's animals were dachshunds, and consisted of two pairs from two litters. One of each pair was killed, and the second allowed to grow for just over 30 days. A comparison of the musculature and the body weights before and after the growth period, showed that for an average gain of 1,698 gm. in body weight, the musculature increased 733 gm., or 1 gm. gain in body weight for 0.43 gm. gain in muscle.

In Table V, is represented the gains in weight, N, and creatinine excretion of our puppies, and from the figures given, and the data of Falck, it is a simple matter to calculate the absorption of creatine due to the increase in the muscular system during growth, as according to the calculations of Bürger, 98 per cent of the total body content of creatine, is contained in that system. We should

TABLE V.

Dog.	Length of experiment.	Weight.			Total N gain.	Creatinine excretion.		
		Initial.	Final.	Gain.		Initial.	Final.	Gain.
	<i>days</i>	<i>kg.</i>	<i>kg.</i>	<i>kg.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
D.....	93	2.30	4.15	1.85	49.50	46	115	69
K.....	112	2.50	4.18	1.68	64.47	48	106	58
L. B.....	140	6.70	11.00	4.30	95.42	180	324	144
Fn.....	90	2.35	4.72	2.37	65.13	47	113	66
Fl.....	80	2.90	5.20	2.30	67.18	59	141	82
Sum of gains.....				12.50	341.70			419

like to point out that the length of time of the experiments (80 to 140 days) with continuous observations on five animals, all of them either absolutely normal or very nearly so, make our figures reliable as average figures for this type of dog. Animals Fn. and Fl. were mongrel terriers, who, though equal to the other three animals in total growth, nevertheless, showed irregular growth periods. For the purpose of averages, however, over a period as long as 3 months, they may be taken as normal. From the figures of Table V the following relationships may be determined.

1 gm. gain N = 36.6 gm. gain body weight = 1.22 mg. gain creatinine excretion.

Coupled with the data of Falck

1 gm. gain body weight = 0.43 gm. gain in musculature.

and the average creatine content of dog muscle = 0.37 per cent.

1 gm. gain body weight	=	1.59 mg. creatine absorption.
1 " " "	=	58 " " "
1 mg. " creatinine excretion	=	48 " " "

In making a choice of which factor, gain in body weight, N, or creatinine excretion, shall be used to calculate the creatine absorption in a particular case, we have chosen that of N gain. Were we dealing with averages instead of particular cases, it would, of course, be immaterial which factor was chosen, but in individual cases, the three methods of judging growth do not always run parallel. Gains in body weight, as they may be due to gains in water or fat, as well as protein, are naturally more subject to fluctuation. Gains in creatinine and N usually correspond much more closely. When, however, the gain in N is very suddenly increased to a high level, then creatinine appears to lag behind. On the whole, it is judged that gains in N represent more closely than the other two, the real anabolism.

In Table VI these calculations are applied to the data upon the experiments on Dogs D, K, and L.B., as shown in Table I. It appears from this, that creatine excretion, either *in toto* or per kilo of body weight, which may either rise or fall on an increase in protein intake, when such an increase is below the level necessary to produce a maximum rate of growth, becomes a creatine production, rising with the level of increasing protein intake until a maximum is reached.

Table VI also contains the total creatine coefficients of the animals under conditions of maximum creatine production. In calculating the total creatine coefficients of children it was sufficient to take the average creatinine and creatine excretion, as the experimental periods were short and there were no appreciable changes in growth during that time. The calculation in puppies must take such changes into account and becomes

$$\frac{\text{Creatine excreted} + \text{creatine absorbed} + \text{creatinine} \times 1.16}{\text{Body weight in kilos}},$$

the creatinine being the average amount excreted at the beginning of, or just previous to the experimental period.

It will be noticed at once that the total creatine coefficients of the three animals do not show very good agreement. If these figures represent real coefficients, evidently the range of variation in the dog is very great. There is reason to believe, however, that in Dog L. B. the true total creatine coefficient is much higher than the figure given in Table VI. In Table III, the total creatine coefficients of Dogs D and K are given. In both animals, diet 8 was given after a short period of fasting; diets 1, 1^x, 2, and 2^x being prior to fasting. It will be noticed that the total creatine coefficients taken as a whole, run at a lower level in a post-fasting feeding period. This is true of all animals who were

TABLE VI.

Dog.	N intake.	Per kg. body weight per day.					Total creatine coefficient.
		N balance.	Creatinine.	Creatine excretion.	Creatine absorbed.	Creatine excretion + creatine absorption.	
	<i>gm.</i>	<i>gm.</i>	<i>m/g.</i>	<i>m/g.</i>	<i>m/g.</i>	<i>m/g.</i>	
D.....	1.33	0.14	21.3	23.8	8.1	31.9	56.5
	2.66	0.34	22.9	32.4	19.7	52.1	78.6
	5.33	0.37	23.3	27.0	21.5	48.5	75.5
K.....	1.33	0.06	19.3	30.9	3.5	34.4	56.8
	2.66	0.33	20.6	23.8	19.1	42.9	66.7
	5.33	0.38	24.2	19.1	22.0	41.1	69.2
L. B.....	2.00	0.00	27.9	11.8	0.0	11.8	44.2
	5.55	0.24	27.6	8.5	13.9	22.4	54.4
	10.88	0.29	27.7	7.6	16.8	24.4	56.5

examined in this way. Thus, the total creatine coefficients of Dog T. B. before fasting were 50.6 and 56.2, whereas after fasting they were 43.8, 35.6, 46.4, 35.6, 46.0, and 39.7, the low figures in this case persisting for 40 days. Dog L. B. had not been fasted, but just previous to the period indicated by the figures of Tables I and VI, it had been on a period of negative N balance for 8 days, this having been brought about by a too sudden diminution in the level of the protein intake. The total creatine coefficient of this animal on an excessively high protein diet prior to the experiments just cited, and on which it was able to make larger gains was found to be 77.7. In Table VII, we have collected the total creatine coefficients of our animals as observed on maximum protein con-

sumption and previous to any fasting or negative N balance. We have included the total creatine coefficients of the same and other animals during a short period of fasting, as it is of decided interest that the figures are of the same magnitude. The fasting periods in all cases were of short duration, and followed one of high protein feeding.

TABLE VII.
Maximum Total Creatine Coefficients.

Dog.	High protein.	Fasting.
D.....	78.6	75.2
K.....	69.2	64.1
L. B.....	77.7	
Fn.....	69.3	
Fl.....		67.8
T. B.....		74.2

TABLE VIII.
Creatinine Coefficients in Dogs.

Dog.	Weight.	Creatinine coefficient.	Dog.	Weight.	Creatinine coefficient.
	<i>kg.</i>			<i>kg.</i>	
D.....	2.50	19.2	Fn.....	2.35	20.0
	3.65	24.4		3.65	24.6
	4.15	27.6		4.65	24.5
K.....	2.50	19.6	Fl.....	2.90	20.3
	3.65	23.9		4.15	22.7
	4.15	25.6		5.20	26.9
L.B.....	6.70	26.2			
	9.45	28.8			
	10.90	29.7			

The height of the total creatine coefficients would indicate that in the dog, the simple relationship of creatinine coefficient, and total creatine coefficient, which was found in man, does not hold. The creatinine coefficient of the adult dog is given by Myers and Fine as 22.5 and by Benedict and Osterberg (12) as 27.6. Our own figures in Table VIII show that the number lies around such a value. The total creatine coefficient is more than twice the value of the creatinine coefficient. Dogs D, K, and L. B., were almost

full grown, and Dog Fn. had reached its maximum creatinine coefficient at the close of our experiments. Nor was there any indication that the amount of creatine excreted was diminishing with the approach of adolescence. Our experiments, however, are not definite enough to disprove such a point. From such figures one would draw the conclusion that creatinuria would persist throughout the life of the dog, at least of the female, as all our animals were of that sex. Such a conclusion would be contrary to the usually accepted fact that the normal adult dog excretes no creatine. Thus, Krause (13), in his experiments on the influence of the estrual cycle, upon creatinuria, observes no creatinuria during the intermenstrual period of a female dog, though it is present during the estrum. There is, however, one point to be noted in our animals. Although they had attained maximum creatinine coefficients, or nearly so, they were still capable of making gains in nitrogen and none of them had reached sexual maturity. In the beginning of this paper, we have stressed the influence of the positive N balance on the height of the excretion and production of creatine. We have also emphasized the effect of fasting or negative N balance upon the level of creatine production as we calculate it. We do not know, however, the effect of a continued period of balance in N metabolism upon creatinuria. We should infer that the result would be a diminution. The very high total creatine coefficient in puppies might represent a factor of safety in creatine production to meet any extraordinary demand which might arise during a period of excessively high rate of growth such as the animal can make under optimum conditions.

SUMMARY.

1. In growing dogs on diets containing increasing amounts of protein, creatine production increases and finally reaches a maximum.
2. The maximum creatine production corresponds to the maximum positive N balance.
3. Creatine excretion in the growing dog under conditions of constant N intake, varies inversely with the height of the N gain.
4. Catabolized protein *per se* cannot be considered as the origin of creatine.

5. Data on the growth of the dog correlating gain in weight, gain in N, and gain in creatinine excretion are given.

6. In the growing dog, as an average figure, it is found that 1 gm. gain in N is correlated with the absorption of 58 mg. of creatine.

7. The total creatine coefficient of puppies is more than twice the value of the creatinine coefficient of the adult dog.

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Protocol 1.*

Dog D.

Date.	Volume.	Diet.	N intake daily.	Daily analysis.				Body weight.	
				Total N of urine.	N balance.	Creat- inine.	Creatine excreted.		
<i>1921</i>	<i>cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>	
Oct. 26	240	1*	1.33	0.77		48	46	2.43	
" 27	210			0.82		48	98	2.45	
" 28	270			1.02		47	115	2.47	
" 29	190			0.92		52	103	2.46	
" 30								2.42	
" 31	125			0.65		44	80	2.46	
Nov. 1	185			1.00		54	108	2.33	
" 2								2.30	
" 3								2.31	
" 4								2.29	
" 5	275				0.92		51	61	2.30
" 6	225				0.78		45	66	2.33
" 7	235				0.73		44	67	2.33
" 8	195				0.85		45	85	2.33
" 9									2.33
" 10	230				0.86		45	102	2.29
" 11	225				0.98		44	114	2.28
" 12	150				0.98		41	137	2.26
" 13	250				1.05		51	117	2.25
Average.....			1.33	0.88	+0.16	47	93	2.35	
Nov. 14	260	1	1.33	0.73		48	66	2.29	
" 15	115			0.72		43	53	2.32	
" 16	170			0.78		49	53	2.35	
" 17	120			0.78		50	52	2.38	
" 18	160			0.92		54	60	2.37	
" 19								2.33	
" 20	205			0.78		48	53	2.33	
" 21	255			0.77		55	61	2.36	
" 22	160			0.73		50	61	2.36	
" 23	165			0.67		50	32	2.39	
" 24	165			0.77		55	47	2.39	
" 25	160			0.87		50	75	2.38	
Average.....			1.33	0.77	+0.33	50	56	2.35	

* In all the protocols, the days on which no analysis appears are the days on which the experimental diet was given, but the animal was allowed freedom for purposes of exercise.

Protocol 1—Concluded.

Date.	Volume.	Diet.	N intake daily.	Daily analysis.				Body weight.
				Total N of urine.	N balance.	Creat- inine.	Creatine excreted.	
<i>1921</i>	<i>cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
Nov. 26	140	2	2.66	1.08		48	93	2.42
" 27	130			1.26		50	90	2.47
" 28	165			1.23		53	65	2.55
" 29								2.59
" 30								2.59
Dec. 1	175	3		1.23		61	41	2.67
" 2	175			1.43		65	84	2.70
" 3	200			1.40		63	107	2.69
" 4	115			1.14		55	78	2.82
" 5	185			1.38		66	77	2.86
" 6	275			1.54		73	94	2.87
" 7	205			1.27		65	79	2.89
" 8	225			1.39		74	128	2.93
" 9	225			1.39		70	117	2.96
Average.....				2.66	1.31	+0.94	62	88
Dec. 10	180	4	5.33	2.55		71	106	3.02
" 11	300			3.36		73	88	3.11
" 12	335			3.45		76	50	3.14
" 13								3.20
" 14	210			3.14		74	44	3.26
" 15	200			3.23		74	53	3.33
" 16								3.33
" 17	280			3.30		77	89	3.41
" 18	250			3.72		84	99	3.48
" 19	220			4.13		90	150	3.52
" 20	190			2.84		75	121	3.53
Average.....				5.33	3.30	+1.23	77	89

Protocol 2.*

Dog K.

Date.	Volume.	Diet.	N intake daily.	Daily analysis.				Body weight.
				Total N of urine.	N balance.	Creat- inine.	Creatine excreted.	
<i>1921</i>	<i>cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
Oct. 26	230	1 ^x	1.33	0.81		48	49	2.51
" 27	195			0.83		51	87	2.52
" 28	170			0.83		45	80	2.56
" 29	185			0.86		53	105	2.56
" 30								2.48
" 31	140			0.77		50	75	2.52
Nov. 1	235			0.71		46	67	2.48
" 2	125			0.78		51	96	2.50
" 3	140			0.70		47	70	2.47
" 4								2.46
" 5	115			0.72		43	73	2.50
" 6	140			0.77		47	66	2.46
" 7	165			0.87		51	66	2.46
" 8	130			0.92		47	80	2.46
" 9								2.46
Average.....			1.33	0.80	+0.16	48	77	2.49
Nov. 10	160	2 ^x	2.66	1.30		51	87	2.48
" 11	150			1.39		53	81	2.51
" 12	120			1.39		41	77	2.50
" 13	145	2		1.41		51	86	2.53
" 14	130		1.36		51	40	2.59	
" 15	135		1.48		51	49	2.59	
" 16	125		1.45		52	37	2.65	
" 17	140		1.54		61	33	2.68	
" 18	140		1.55		60	66	2.67	
" 19							2.68	
" 20	185		1.45		61	44	2.70	
" 21	110		1.22		58	66	2.76	
" 22	135		1.39		57	75	2.77	
Average.....			2.66	1.41	+0.85	54	62	2.62
Nov. 23	110	2	2.66	1.39		55	100	2.78
" 24	155			1.56		62	102	2.78
" 25	110			1.49		60	72	2.83
" 26	160			1.70		59	86	2.81

Protocol 2—Concluded.

Date.	Volume.	Diet.	N intake daily.	Daily analysis.				Body weight.
				Total N of urine.	N balance.	Creat- inine.	Creatine excreted.	
<i>1921</i>	<i>cc.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
Nov. 27	135	3		1.70		62	84	2.82
“ 28	135			1.54		62	91	2.88
“ 29								2.90
“ 30								2.91
Dec. 1	120			1.48		63	54	2.98
“ 2	165			1.57		75	87	2.96
“ 3	125			1.64		65	115	2.94
“ 4	105			1.34		62	82	3.03
“ 5	155			1.50		72	82	3.05
“ 6	140			1.34		65	88	3.10
Average.....			2.66	1.52	+0.69	64	87	2.91
Dec. 7	185	4	5.33	2.76		72	74	3.12
“ 8	225			2.95		79	58	3.24
“ 9								3.16
“ 10	150			2.66		81	56	3.25
“ 11	225			3.29		83	54	3.39
“ 12	320			3.70		82	35	3.39
“ 13	215			3.10		78	38	3.36
“ 14	160			3.50		74	52	3.42
“ 15	175			3.31		81	74	3.49
“ 16								3.30
“ 17	225			3.44		82	119	3.40
“ 18	220			3.30		82	66	3.45
“ 19	240			3.93		84	84	3.51
“ 20	215			3.16		90	64	3.45
Average.....			5.33	3.26	+1.27	81	64	3.35

Protocol 3.*

Dog L. B.

Date.	Volume.	pH	Diet.	N intake daily.	Daily analysis.				Body weight.
					Total N of urine.	N balance.	Creatinine.	Creatinine excreted.	
<i>1921</i>	<i>cc.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
July 21	925	8.4	5	2.00	2.07		271	70	9.16
" 22	955	8.4			1.80		249	123	9.14
" 23	845	8.4			1.65		258	142	9.15
" 24	910	8.4			1.51		242	98	9.15
" 25									9.15
Average.....				2.0	1.76	0.00	255	108	9.15
July 26			6	5.55					9.14
" 27	650	7.4			2.29		226	100	
" 28	800	7.4			2.82		272	79	9.41
" 29	880	7.4			1.96		238	27	9.36
" 30									9.44
" 31									9.45
Aug. 1	875	7.4			2.61		275	80	9.44
" 2	830	7.4			2.56		282	113	9.46
Average.....				5.55	2.45	+2.27	259	80	9.39
Aug. 3	830	8.0	7	10.88	5.73		263	168	9.67
" 4	755	8.0			6.20		272	74	9.84
" 5	955	8.0			6.70		274	45	10.02
" 6	815	8.0			6.90		292	19	10.16
Average.....				10.88	6.38	+2.87	275	76	9.92

*Protocol 4.**

Dog D, diet S, N intake 4.30 gm.

Date.	Volume.	Daily analysis.				Body weight.
		Total N of urine.	N balance.	Creatinine.	Creatine excreted.	
<i>1922</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
Jan. 15						3.42
" 16	105	2.48		70	35	3.52
" 17	175	3.28		108	24	3.62
" 18	115	2.39		88	16	3.68
" 19	90	2.08		92	17	3.77
" 20	115	2.37		95	19	3.76
" 21	180	2.18		84	12	3.82
" 22						3.80
" 23	145	2.39		78	18	3.88
" 24	170	2.40		122	38	3.93
Average.....		2.45	+1.15	92	22	3.72
Jan. 25	135	2.58		97	61	3.94
" 26	215	3.04		110	150	3.98
" 27	170	3.03		106	130	3.95
" 28	125	2.96		95	107	3.95
" 29	155	3.80		117	87	3.98
" 30	160	3.60		99	46	3.93
" 31	195	3.96		126	43	3.92
Feb. 1	170	3.27		113	42	4.03
" 2	160	3.34		106	72	4.06
" 3	195	3.32		115	83	4.05
" 4	150	3.06		108	112	4.10
Average.....		3.27	+0.33	108	85	3.99
Feb. 5						4.02
" 6	105	2.80		105	108	4.13
" 7	155	3.24		122	184	4.03
" 8	190	3.42		119	175	4.06
" 9						4.10
" 10	125	2.79		108	107	4.12
" 11	335	3.76		128	80	4.09
" 12	165	3.13		112	82	4.12
" 13	95	2.70		101	84	4.16
" 14	145	3.36		127	130	4.17
Average.....		3.15	+0.20	115	119	4.10

*Protocol 5.**

Dog K, diet 8, N intake 4.30 gm.

Date.	Volume.	Daily analysis.				Body weight.
		Total N of urine.	N balance.	Creatinine.	Creatine excreted.	
<i>1922</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>kg.</i>
Jan. 15						3.30
" 16	145	3.16		80	51	3.39
" 17	165	2.89		79	33	3.42
" 18	125	2.65		83	32	3.54
" 19	210	2.82		91	30	3.60
" 20	170	2.86		85	24	3.56
" 21	115	2.46		85	15	3.65
" 22						3.62
Average.....		2.81	+0.78	84	31	3.51
Jan. 23	120	2.70		92	9	3.71
" 24	80	2.28		84	9	3.73
" 25	155	2.58		86	20	3.74
" 26	115	2.36		88	9	3.75
" 27	185	2.86		97	15	3.80
" 28	85	2.50		77	6	3.87
" 29	90	2.33		75	11	3.90
" 30						3.86
" 31	130	2.67		89	11	3.92
Feb. 1	140	2.77		110	8	3.98
" 2	90	2.38		91	15	4.06
" 3	200	2.58		104	24	4.08
" 4	130	2.38		95	20	4.05
" 5	125	2.53		108	10	4.12
" 6	110	2.58		105	33	4.19
Average.....		2.53	+1.01	93	14	3.92
Feb. 7	115	2.88		99	39	4.10
" 8	165	2.93		105	64	4.12
" 9	140	3.05		104	80	4.10
" 10	140	2.89		102	65	4.13
" 11	140	3.00		108	34	4.14
" 12	210	2.98		102	30	4.14
" 13	85	2.32		95	16	4.14
" 14	130	3.18		132	35	4.18
Average.....		2.90	+0.51	106	45	4.14

ALKALOSIS VERSUS ABNORMAL SODIUM ION CONCENTRATION AS A CAUSE OF TETANY.

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The popularity of sodium bicarbonate therapy in pediatric practice has in recent years led to the recognition of numerous cases of tetany in children (1), presumably brought on by an excessive administration of the drug; and even a few examples of adult tetany apparently referable to an excessive administration of sodium bicarbonate have been reported in the literature (2).

Two views as to the cause of "bicarbonate tetany" have been advanced. According to Van Slyke (3) tetany is observed when the hydrogen ion concentration of the blood has attained a pH of about 7.8, whether this abnormal hydrogen ion concentration is caused by an uncompensated CO_2 deficit or by an uncompensated alkali excess. Greenwald (4), however, maintains that the part played by alkalosis is a distinctly negligible factor, that the cause of tetany in these cases is the presence in the body of a preponderatingly large amount of sodium salts; in other words, that tetany following the administration of large amounts of sodium bicarbonate is a demonstration of the poisonous effect of the sodium ion. In proof of his view Greenwald has carried out many experiments on dogs in which he has shown that convulsions may be produced not only by the intravenous injection of large amounts of sodium bicarbonate but also by the administration of sodium chloride, phosphate, or sulfate.

In the summary to his last paper on the subject Greenwald makes the following statement:

"At the time convulsions appear, the concentration of sodium in the plasma is the same as was observed after the injection of other sodium

salts (chloride, sulfate, phosphate). The convulsions are ascribed to 'sodium poisoning,' a disturbance, due to an excess of sodium, of the normal equilibrium between sodium and other ions."

According to the views most commonly accepted the appearance of tetany is indicated by an increased irritability of the motor and sensory nerves to electrical or mechanical stimulation, and it is, we believe generally conceded by both clinicians and physiologists that it is practically impossible to state with absolute certainty whether convulsions observed in man or in animals are or are not due to tetany without a determination of the electrical reactions. As apparently most of the investigators who have worked on "sodium bicarbonate tetany" have not taken the precautions to make observations of electrical excitability it has seemed to us worth while to repeat some of the work involving the injection of sodium salts and in addition to observe the response of these animals to electrical stimulation.

Dogs were used throughout the investigation. These animals were brought to the laboratory at least 1 week before operation, and only animals who appeared to be in good health were used. Our experiments were all carried out during the morning hours on animals who had had no food since noon of the previous day.

The general experimental procedures used were as follows:

After the animal had been fastened to an animal holder the inner surface of the leg and the abdomen were shaved. Ether was then administered, and as soon as anesthesia was sufficiently deep a tracheal cannula was inserted and a sample of blood (20 to 50 cc.) was removed from the carotid artery. A solution of the salt under investigation was then injected into the external jugular vein at intervals as indicated in the protocols of the individual experiments. Further samples of blood were removed from the carotid artery at appropriate times, and finally the electrical reactions of the peroneal nerve were determined before the administration of salt solutions and at approximately 15 minute intervals thereafter until the conclusion of the experiment.

At each withdrawal of blood two samples were taken; the first being received under mineral oil in a tube containing a little powdered potassium oxalate was used for the determination of the CO_2 content by the method of Van Slyke and Cullen (5) and of the pH by the colorimetric procedure of Cullen (6), the second was removed with a syringe, allowed to clot in the refrigerator, and used for the determination of sodium by the method of Kramer and Tisdall (7), and of calcium by the procedure of Clark (8).

The electrical reactions were determined with a Victor galvanic wall plate to which a specially built milliammeter was attached. This milliam-

meter has a range of from 0 to 10 milliamperes and is graduated in 0.1 milliampere. The stimulating electrode was of the globule Stinzig type, while the indifferent electrode was constructed of a wide band of copper to one margin of which a binding post had been attached, the electrode being covered with wadding so as to absorb the salt solution. This electrode was placed around the shaved abdomen of the animal and the edges were tied fairly tightly together. The peroneal nerve in the leg was sought and having been located, the Stinzig electrode was not moved until each group of reactions had been completed. Salt solution was used to keep the electrodes moist. Reactions which are considered characteristic of tetany or spasmophilia are those showing either anodal reversal ($AOC < ACC$ and < 5 milliamperes) or $COC < 5$ milliamperes or $CCTe = 5$ milliamperes.

Reactions showing $AOC < 5$ milliamperes but $> ACC$ are suggestive of approaching hyperirritability, but are not considered of the spasmophilic type by the majority of workers in this field.

Experiment 1.

Administration of Sodium Bicarbonate.

Dec. 20, 1922. Dog 1, male, weight 6 kg., a very young animal.

10.05 a.m. Ether administration started.

10.15-10.20 a.m.	CCC	ACC	AOC	COC
	1.0	4.4	5.7	7.5

10.45 a.m. 40 cc. blood taken from the carotid artery (Sample 1).

10.50 a.m.	CCC	ACC	AOC	COC
	1.0	4.3	>5	>5

11.20-11.30 a.m. Injected 50 cc. 10 per cent $NaHCO_3$ solution.

11.30 a.m.	CCC	ACC	AOC	COC
	1.5	6.2	>10	>10

11.35 a.m. 50 cc. blood taken from the carotid artery (Sample 2).

11.40 a.m.	CCC	ACC	AOC	COC
	1.7	6.7	>10	>10

11.52 a.m.	CCC	ACC	AOC	COC
	2.5	6.6	>10	>10

11.45-11.55 a.m. Injected 50 cc. 10 per cent $NaHCO_3$ solution; at 11.50 a.m. respiration became much slower.

12.10 p.m. 50 cc. blood taken from the carotid artery (Sample 3).

12.20 p.m.	CCC	ACC	AOC	COC
	1.5	3.5	2.7	9.0

The total amount of sodium bicarbonate injected was 15 gm. which is equivalent to 2.5 gm. per kg. (0.67 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		vol. per cent	mg. per 100 cc.	mg. per 100 cc.
1	7.4	42.8	361	10.3
2	7.45	78.0	419	10.4
3	7.6	94.6	478	10.4

*Experiment 2.**Administration of Sodium Bicarbonate.*

Dec. 29, 1922. Dog, 2, male, weight 16 kg.

10.00 a.m. Ether administration started.

10.30 a.m.	CCC	ACC	AOC	COC
	2.5	6.7	>10	>10

10.50 a.m. 40 cc. blood taken from the carotid artery (Sample 1).

11.05 a.m.	CCC	ACC	AOC	COC
	3.2	7.0	>10	>10

11.30–11.32 a.m. Injected 50 cc. 10 per cent NaHCO₃ solution into external jugular vein.

11.45 a.m.	CCC	ACC	AOC	COC
	3.5	8.0	>10	>10

12.10 p.m. 40 cc. blood taken from carotid artery (Sample 2).

12.20–12.24 p.m. Injected 100 cc. 10 per cent NaHCO₃ solution into external jugular vein.

12.30 p.m.	CCC	ACC	AOC	COC
	4.5	9.0	>10	>10

12.50 p.m. Complete failure of respiration, 40 cc. blood removed from the heart (Sample 3).

The total amount of sodium bicarbonate injected was 15 gm. which is equivalent to 0.93 gm. per kg. (0.25 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		vol. per cent	mg. per 100 cc.	mg. per 100 cc.
1	7.4	42.8	371	9.3
2	7.44	57.9	451	8.6
3	7.5	86	540	7.3

*Experiment 3.**Administration of Sodium Bicarbonate.*

Jan. 10, 1923. Dog 3, male, weight 9 kg.

9.10 a.m. Ether administration started.

9.40 a.m.	CCC	ACC	AOC	COC
	1.0	4.0	7.3	10

10.00 a.m. 50 cc. blood taken from the carotid artery (Sample 1).

10.09–10.15 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution into the external jugular vein.

10.18 a.m.	CCC	ACC	AOC	COC
	1.2	5.0	>10	>10

10.40 a.m. 50 cc. blood taken (Sample 2).

10.50 a.m.	CCC	ACC	AOC	COC
	1.7	5.4	>10	>10

10.58–11.01 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

11.02–11.06 a.m. Duplicated the above injections.

11.04–11.14 a.m.	CCC	ACC	AOC	COC
	1.6	4.5	5.4	>10

11.25 a.m. 25 cc. blood taken (Sample 3).

11.31–11.37 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

12.00–12.04 p.m.	CCC	ACC	AOC	COC
	1.5	3.5	4.4	>10

12.15 p.m. 50 cc. blood taken (Sample 4).

1.00 p.m.	CCC	ACC	AOC	COC
	1.0	3.3	2.5	9.0

3.35 p.m. Respiration failed and a final sample of blood was taken from the heart (Sample 5).

The total amount of sodium bicarbonate injected was 20 gm. which is equivalent to 2.2 gm. per kg. (0.59 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		<i>vol. per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	7.38	42.4	367	12
2	7.46	60.4	379	10.2
3	7.52	103.8	474	9.8
4	7.52	107.6	595	9.3
5	7.50		332	9.3

*Experiment 4.**Administration of Sodium Bicarbonate.*

Jan. 25, 1923. Dog 4, male, weight 12 kg.

8.10 a.m. Ether administration started.

9.00 a.m.	CCC	ACC	AOC	COC
	2.6	5.3	>10	>10

9.35 a.m.	CCC	ACC	AOC	COC
	1.3	6.5	4.5	>10

9.40 a.m. 20 cc. blood taken from the carotid artery (Sample 1).

10.00 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution into the external jugular vein.

10.12 a.m.	CCC	ACC	AOC	COC
	1.2	3.1	4.0	>10

10.22–10.23 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

10.30 a.m.	CCC	ACC	AOC	COC
	1.0	3.0	3.5	>10

10.35–10.37 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

10.50 a.m.	CCC	ACC	AOC	COC
	1.0	3.1	2.6	>10

10.55–10.56 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

11.15 a.m.	CCC	ACC	AOC	COC
	0.7	3.5	2.6	>10

11.33–11.35 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

12.05 p.m.	CCC	ACC	AOC	COC
	1.0	4.0	3.5	CCTe6

12.25 p.m. 45 cc. blood taken from the carotid artery (Sample 2).

3.00 p.m.	CCC	ACC	AOC	COC
	1.3	3.5	4.4	CCTe 4.5

3.15 p.m. 40 cc. blood taken from carotid artery (Sample 3).

The total amount of sodium bicarbonate injected was 25 gm. which is equivalent to 2.0 gm. per kg. (0.54 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		vol. per cent	mg. per 100 cc.	mg. per 100 cc.
1	7.4	33.4		12.0
2	7.58	96.2	444	10.1
3	7.44	69.2	463	9.6

*Experiment 5.**Administration of Sodium Bicarbonate.*

Mar. 19, 1923. Dog 5, young male, weight 10.2 kg.

8.50 a.m. Administration of ether started.

8.55 a.m.	CCC	ACC	AOC	COC
	3.5	6.2	>10	>10

9.10 a.m. 50 cc. blood taken from the carotid artery (Sample 1).

9.20-9.21 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution into the external jugular vein.

9.35 a.m.	CCC	ACC	AOC	COC
	3.0	6.5	6.0	>10

9.41-9.43 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

9.55 a.m.	CCC	ACC	AOC	COC
	3.0	6.5	3.5	>10

10.03-10.05 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

10.20 a.m.	CCC	ACC	AOC	COC
	2.5	5.5	3.0	>10

10.27-10.29 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

10.35 a.m. 50 cc. blood taken (Sample 2).

11.21-11.24 a.m. Injected 50 cc. 10 per cent NaHCO_3 solution.

11.25 a.m.	CCC	ACC	AOC	COC
	2.5	5.4	3.0	9.0

11.42 a.m. Respiration which had become very slow and shallow ceased entirely.

11.44 a.m. 50 cc. blood taken from the heart (Sample 3).

The total amount of sodium bicarbonate injected was 25 gm. which is equivalent to 2.4 gm. per kg. (0.658 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		<i>vol. per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	7.4	41.4	347	10.8
2	7.55	98.2	528	9.0
3	7.52	97.0	481	7.9

*Experiment 6.**Administration of Sodium Chloride.*

Feb. 12, 1923. Dog 6, male, weight 14 kg.

9.00 a.m. Administration of ether started.

9.30 a.m.	CCC	ACC	AOC	COC
	1.0	>10	>10	>10

10.12 a.m. 40 cc. blood taken from the carotid artery (Sample 1).

10.15 a.m.	CCC	ACC	AOC	COC
	1.6	3.0	>5	>5

10.20-10.26 a.m. Injected 50 cc. 10 per cent NaCl solution into the external jugular vein.

10.30 a.m.	CCC	ACC	AOC	COC
	3.0	4.3	>10	>10

10.36-10.38 a.m. Injected 50 cc. 10 per cent NaCl solution.

10.45 a.m.	CCC	ACC	AOC	COC
	3.0	4.5	>10	>10

11.00 a.m.	CCC	ACC	AOC	COC
	3.3	4.4	>10	>10

11.04-11.06 a.m. Injected 50 cc. 10 per cent NaCl solution.

11.08 a.m.	CCC	ACC	AOC	COC
	3.6	4.0	>10	>10

11.25 a.m. 40 cc. blood withdrawn (Sample 2).

11.30 a.m.	CCC	ACC	AOC	COC
	4.4	9.5	>10	>10

11.32-11.34 a.m. Injected 50 cc. 10 per cent NaCl solution.

11.55 a.m. Spasmodic twitching of the muscles was noted.

12.12 p.m. 40 cc. blood taken (Sample 3).

12.20 p.m.	CCC	ACC	AOC	COC
	5.5	7.0	>10	>10

3.00 p.m.	CCC	ACC	AOC	COC
	7.5	>10	>10	>10

3.15 p.m. 50 cc. blood taken (Sample 4).

3.25 p.m. Animal killed.

The total amount of sodium chloride injected was 20 gm. which is equivalent to 1.4 gm. per kg. (0.54 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca	Cl
		vol. per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	7.4	55.0	370	12.1	388
2	7.4	30.5	441	12.1	465
3	7.36	20.2	441	11.9	472
4	7.36	14.5	433	10.3	469

*Experiment 7.**Administration of Sodium Chloride.*

Feb. 21, 1923. Dog 7, female, weight 15 kg.

8.45 a.m. Administration of ether started.

9.35 a.m.	CCC	ACC	AOC	COC
	2.0	3.6	>10	>10

9.45 a.m. 50 cc. blood taken from the carotid artery (Sample 1).

9.55-9.59 a.m. Injected 50 cc. 10 per cent NaCl solution into the external jugular vein.

10.00 a.m.	CCC	ACC	AOC	COC
	2.5	5.5	>10	>10

10.08-10.10 a.m. Injected 50 cc. 10 per cent NaCl solution.

10.20 a.m.	CCC	ACC	AOC	COC
	4.0	6.5	>10	>10

10.23-10.25 a.m. Injected 50 cc. 10 per cent NaCl solution.

10.38 a.m.	CCC	ACC	AOC	COC
	4.0	6.5	>10	>10

10.41-10.42 a.m. Injected 50 cc. 10 per cent NaCl solution.

10.50 a.m. Heart stopped beating. 50 cc. blood taken from the heart (Sample 2).

The total amount of sodium chloride injected was 20 gm. which is equivalent to 1.3 gm. per kg. (0.50 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	CO ₂	Na	Ca	Cl
	vol. per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	48.8	372	11.2	382
2	21.1	464	10.4	475

*Experiment 8.**Administration of Sodium Sulfate.*

Feb. 5, 1923. Dog 8, young male, weight 7 kg.

8.30 a.m. Administration of ether started.

8.50 a.m.	CCC	ACC	AOC	COC
	1.6	3.0	> 10	> 10

9.15 a.m. 50 cc. blood taken from the carotid artery (Sample 1).

9.26-9.29 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution (anhydrous) into the external jugular vein.

9.40 a.m.	CCC	ACC	AOC	COC
	1.6	3.3	4.7	> 10

9.46-9.48 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

9.55 a.m.	CCC	ACC	AOC	COC
	1.8	3.5	4.5	> 10

10.01-10.03 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

10.10 a.m. Urine, which appeared to be extremely dilute, began to drip from the urethra, and continued to do so during the remainder of the experiment.

10.15 a.m.	CCC	ACC	AOC	COC
	1.7	4.0	4.0	> 10

10.18-10.20 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

10.40 a.m.	CCC	ACC	AOC	COC
	2.5	5.2	8.5	> 10

10.42-10.44 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

10.45 a.m. Respiration very slow, pulse good, no eye reflex, but fore-legs began to shake, and a general twitching and jerking of all the muscles of the body were noted.

10.55 a.m. 50 cc. blood taken (Sample 2).

10.58 a.m. No eye reflex, the muscular spasms had stopped.

11.00 a.m.	CCC	ACC	AOC	COC
	3.0	6.5	9.0	> 10

11.05 a.m. Muscular twitchings and jerkings had returned, there was violent twitching of the whiskers, no eye reflex.

11.09 a.m. Another convulsive seizure had begun and lasted about half a minute. In the intervals between convulsions the respirations were very rapid.

11.13 a.m. Another convulsive seizure had begun.

11.40 a.m. Animal quiet.

CCC	ACC	AOC	COC
3.0	4.6	7.5	>10

11.50 a.m. Injected 20 cc. 10 per cent CaCl_2 solution subcutaneously into two areas in the thigh.

12.10 p.m. Injected 15 cc. 10 per cent CaCl_2 solution into the femoral vein. The convulsions continued at intervals and were apparently unaffected by the administration of the calcium salt.

12.20 p.m. 50 cc. blood taken (Sample 3). The animal which still continued to have convulsions at intervals was killed at 12.35 p.m.

The total amount of sodium sulfate injected was 25 gm. which is equivalent to 3.5 gm. per kg. (1.16 gm. Na per kg.).

Analysis of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca	Remarks.
		<i>vol. per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
1	7.4	42	391	12.1	
2	7.32	27.7	572	8.9	
3	7.28	16.4	539	30.6	After CaCl_2 injection intravenously.

Experiment 9.

Administration of Sodium Sulfate.

Mar. 14, 1923. Dog 9, male, weight 8.1 kg.

8.35 a.m. Administration of ether started.

8.50 a.m.	CCC	ACC	AOC	COC
	1.7	4.2	8.2	>10

9.05 a.m. 50 cc. blood taken from the carotid artery (Sample 1).

9.11-9.13 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution into the external jugular vein.

9.30 a.m.	CCC	ACC	AOC	COC
	1.2	4.3	6.5	>10

9.32-9.35 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

9.45 a.m.	CCC	ACC	AOC	COC
	1.6	3.8	4.2	>10

9.53-9.55 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

10.10 a.m.	CCC	ACC	AOC	COC
	2.0	4.5	6.5	>10

10.14-10.16 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

10.15 a.m. Respirations became very slow.

10.25 a.m. 50 cc. blood taken (Sample 2).

10.51-10.55 a.m. Injected 50 cc. 10 per cent Na_2SO_4 solution.

11.55 a.m. Dog died of respiratory failure.

The total amount of sodium sulfate injected was 25 gm. which is equivalent to 3.1 gm. per kg. (1.03 gm. Na per kg.).

Analyses of Serum and Plasma.

Sample No.	pH	CO ₂	Na	Ca
		vol. per cent	mg. per 100 cc.	mg. per 100 cc.
1	7.35	30.5	321	10.9
2	7.32	23.3	491	9.9

Controls.

In order to study the effect of prolonged etherization on the electrical excitability of the peripheral nerves the following two experiments were carried out.

Experiment 10.

Feb. 5, 1923. Dog 10, female, weight 4 kg.

9.15 a.m. Ether administration begun.

9.45 a.m.	CCC	ACC	AOC	COC
	1.0	3.0	5.0	5.5

10.05 a.m. 30 cc. blood withdrawn from carotid artery.

11.00 a.m.	CCC	ACC	AOC	COC
	1.2	3.0	4.9	5.5

12.00 m.	CCC	ACC	AOC	COC
	1.2	2.9	5.5	>10

1.30 p.m. Dog died.

Experiment 11.

Feb. 8, 1923. Dog 11, male, weight 7.8 kg.

8.50 a.m. Ether begun.

9.35 a.m.	CCC	ACC	AOC	COC
	3.2	5.0	>10	>10
10.35 a.m.	CCC	ACC	AOC	COC
	2.5	5.6	>10	>10

11.00 a.m. Dog died.

A summary of Experiments 1 to 11 inclusive is given in Table I.

It will be noted that with the exception of Experiment 2, in which the dosage of sodium bicarbonate was low, all the experiments in which this salt was injected resulted in the attainment of a condition of hyperirritability as shown by the spasmophilic reactions, which were invariably observed. On the other hand, in the two experiments in which sodium chloride was injected the electrical reactions showed a constant decrease in irritability, although serum sodium rose 19 and 24 per cent, respectively, and the calcium showed a slight decrease. In the two experiments in which sodium sulfate was injected it was noted that after two or three injections of the salt the reactions approached the spasmophilic type, but on the injection of more sodium sulfate they again became normal. In both the sodium chloride and the sodium sulfate experiments marked acidosis developed as shown by the fall in CO_2 content of the plasma, and in all these experiments there was also a fall in the calcium concentrations, which latter finding is difficult to explain except on the theory of dilution of the blood by the large volumes of fluid injected.

With the exception of Experiments 2, 10, and 11 there invariably occurred, sometimes after two or three injections of salt, sometimes only after the entire amount had been given, various twitching and jerking movements, frequently associated with great rigidity of the limbs, which might well be termed "convulsions," but which in the case of the dogs injected with sodium chloride and sodium sulfate were accompanied by electrical reactions which were absolutely normal.

Our results are therefore confirmatory of the findings of Greenwald, as regards the production of convulsive movements in dogs

TABLE I.
Summary of Experiments 1 to 11.

Experiment No.	Salt used.	Na	Remarks.
1	NaHCO ₃	<i>gm. per kg.</i> 0.67	Reactions taken after the last portion of NaHCO ₃ solution had been injected were of spasmophilic type. Examination of serum showed no change in calcium; sodium rose from 361 to 478 mg.; pH from 7.4 to 7.6; and CO ₂ from 42.8 to 94.6 vol. per cent.
2	NaHCO ₃	0.25	Reactions show a constant <i>decrease</i> in irritability. Examination of serum showed a decrease of calcium from 9.3 to 7.3 mg.; sodium rose from 371 to 540 mg.; pH from 7.4 to 7.5; and CO ₂ from 42.8 to 86 vol. per cent.
3	NaHCO ₃	0.59	Observations taken after final injection of NaHCO ₃ showed reactions of spasmophilic type. Examination of serum showed a decrease in calcium from 12 to 9.3 mg.; a rise in sodium from 367 to 595 mg.; in pH from 7.38 to 7.5; and in CO ₂ from 42.4 to 107.6 vol. per cent.
4	NaHCO ₃	0.54	Reactions taken after the last injection of sodium bicarbonate showed definite spasmophilia. The serum calcium fell from 12 to 9.6 mg.; the pH rose from 7.4 to 7.58; and the CO ₂ from 33.4 to 96.2 vol. per cent.
5	NaHCO ₃	0.65	Reactions taken after the last injection of sodium bicarbonate showed definite spasmophilia. The serum calcium was found to have fallen from 10.8 to 7.9 mg.; the sodium rose from 347 to 528 mg.; pH from 7.4 to 7.52; and CO ₂ from 41.4 to 98.2 vol. per cent.
6	NaCl	0.54	Reactions showed a constant decrease in irritability. The serum calcium was decreased from 12.0 to 10.3 mg.; sodium increased from 370 to 441 mg.; pH decreased from 7.4 to 7.36; and CO ₂ from 55.0 to 14.5 vol. per cent.

TABLE I—*Concluded.*

Experiment No.	Salt used.	Na <i>gm. per kg.</i>	Remarks.
7	NaCl	0.50	Reactions showed a constant decrease in irritability. The serum calcium decreased from 11.2 to 10.4 mg.; sodium increased from 372 to 464 mg.; and CO ₂ decreased from 48.8 to 21.1 vol. per cent.
8	Na ₂ SO ₄	1.16	Reactions taken at 10.15 a.m. after the third injection of Na ₂ SO ₄ approached the spasmodic type, but never definitely reached it. Those taken after the final injection were normal. Serum calcium decreased from 12.1 to 8.9 mg.; sodium increased from 391 to 572 mg.; pH decreased from 7.4 to 7.28; and CO ₂ from 42 to 16.4 vol. per cent.
9	Na ₂ SO ₄	1.03	Reactions taken at 9.45 a.m. after the second injection of Na ₂ SO ₄ approached the spasmodic type. At 10.10 a.m. (after the third injection) they were normal. The serum calcium fell from 10.9 to 9.9 mg.; sodium increased from 321 to 491 mg.; and CO ₂ decreased from 30.5 to 23.3 vol. per cent.
10	Control.		Reactions obtained at intervals for 3 hours on an etherized dog showed no striking variations.
11	Control.		Reactions obtained at intervals for a period of 2 hours on an etherized dog showed no striking variations.

by the injection of large quantities of sodium bicarbonate, sulfate, and chloride, but we are entirely unable to confirm or agree with the conclusions of this author regarding the point that these convulsions are invariably those of true tetany, brought on by the pressure of an excess of the sodium ion. We have done no work with sodium phosphate as Jeppsson (9) has recently published the

results of an extensive series of experiments in which he tested the electrical reactions after the administration of a series of sodium and potassium salts. As a result of this work he is able to state definitely that of the sodium salts used only the disodium phosphate was active in producing a spasmophilic condition. A somewhat similar result was obtained by Tisdall (10) who found that dogs which received injections of phosphoric acid showed no ill effects while those which were given disodium phosphate showed muscular twitchings which this author interprets as suggestive of incipient tetany. Unfortunately, data regarding the electrical reactions are not included in this latter work. We feel that the experimental results described in this paper give support to the theory that the spasmophilic condition which sometimes follows the excessive ingestion of sodium bicarbonate is probably due to an abnormal $\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}$ ratio (11) and *not* to an abnormal ratio between the calcium and sodium ions.

The suggestion has frequently been made that a decrease in the hydrogen ion concentration of the serum might result in a decreased ionization of serum calcium, a fact which must be considered in connection with the interpretation of the results presented in this paper.

Brinkman (12) and Brinkman and Van Dam (13) have recently published observations on the calcium ion concentration in serum and emphasize strongly the importance of its exact maintenance. According to these authors this maintenance of calcium ion concentration depends on the ratio between the concentration of hydrogen and bicarbonate ions.

More recently one of us together with Pappenheimer, Zucker, and Murray (14) published results obtained in dialysis of serum calcium where the serum and dialyzing fluid were saturated with CO_2 mixtures, the saturation of the serum varying between 17 and 62 mm. of mercury tension. Such a wide range of CO_2 saturation, while, of course, altering the pH of the fluid (from 7.6 to 7.2,) did not result in an increased ionization of the serum calcium as shown by the percentage of diffusible calcium.

SUMMARY.

In a series of experiments which was planned to throw light on the cause of the tetany following excessive sodium bicarbonate administration it was found that when sodium bicarbonate was introduced into dogs by intravenous injection there followed, when the dosage was sufficiently large, various twitching and jerking movements, together with an increased irritability to electrical stimuli. Examination of the blood serum indicated a slight lowering of the calcium content, and an increase in the sodium, CO_2 , and pH.

When sodium chloride or sodium sulfate was injected in place of the bicarbonate, "convulsions" similar to those noted above invariably appeared, but a determination of the electrical reactions showed that in these animals a normal or a decreased irritability prevailed. Examination of the blood serum showed an increased sodium content and a decrease in calcium, CO_2 , and pH.

Our results therefore make it seem improbable that the spasmophilic condition following excessive sodium bicarbonate injection is due to "sodium poisoning," but rather to an abnormal

$\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}$ ratio.

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GLUCOKININ.

SECOND PAPER.

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INTRODUCTION.

In preliminary papers¹ and in an abstract² on this subject a number of results were published which showed that from a variety of plants widely divergent in character extracts could be prepared which had the property when administered to normal rabbits by subcutaneous injection of producing a condition of hypoglycemia. Also, examples were given of a marked decrease in the blood sugar and the sugar excretion observed over a period following the administration of certain plant extracts in depancreatized dogs. The hypoglycemia which was observed in these early experiments was not often very marked, nor was there any absolute agreement in various experiments between the time of its onset and the time of its duration. There was, however, great uniformity in practically all the early experiments in this respect, namely that after a satisfactory method of extraction had been found (CO₂ snow-freezing, hot water extraction, and partial purification with alcohol), hypoglycemia of varying degrees was noted in almost every instance where a plant extract was administered to a normal rabbit.

As the experimental work along these lines was continued a large number of what were termed at the time negative results were obtained. Many normal rabbits receiving plant extracts by

¹ Collip, J. B., *J. Biol. Chem.*, 1923, lvi, 513; *Tr. Roy. Soc. Canada*, 1923, in press.

² Collip, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 321.

subcutaneous injection failed to manifest any marked hypoglycemia during the next few days following the injection. In many instances where such "negative" results were obtained the blood sugar curve was followed for as long as 5 days after the injection, blood sugar analysis being made two to five times daily, and the animals meanwhile being kept without food.

In the early work it was found that extracts representing from 50 to 200 gm. of plant material usually produced a certain degree of hypoglycemia which was followed subsequently by a return to the normal blood sugar level. In a number of experiments extracts equivalent to larger amounts of plant material were injected. Marked toxic action was manifested in these instances and the animal would die within a few hours of the injection. Such toxic action was not met with to any extent in the earlier work where smaller amounts of plant tissue were used in the making of any one extract.

Further investigation showed also that there was a great difference in the type of reaction of different rabbits to the same extract.

The problem has therefore presented many new aspects as the investigation has proceeded. It is chiefly to these that attention will be drawn in this communication.

Methods.

As the starting point of this investigation was the demonstration of the hormone in yeast it may be stated that the first essential in the making of a potent extract from yeast is the thorough disintegration of the yeast cell or the rendering of the same freely permeable. Prolonged grinding in a ball mill or sudden freezing and thawing as with CO₂ snow and hot water were found to be suitable procedures.

Potent plant extracts were first made by freezing to a solid state the fresh tissue with CO₂ snow, grinding to a powder while still frozen, and then dropping this powder into boiling water, the addition of the powder being in such an amount that the temperature was kept between 70 and 80°C. in the mixture. This temperature was maintained for 3 minutes. The mixture was then chilled and added at once to 5 volumes of 95 per cent acetone-denatured alcohol. After filtration the clear green liquor

was concentrated to small volume in a warm air current. When the alcohol was removed by evaporation the chlorophyll was precipitated out as a velvet mat on the tray bottom and a clear yellow, syrupy liquid remained which could readily be filtered off.

Later it was found that potent extracts could be made either by cold alcohol extraction or by refluxing a mixture of powdered plant tissue with 5 volumes of 95 per cent acetone-denatured alcohol.

The preliminary procedure which is now employed consists in rendering the tissue permeable by autoclaving for a few minutes (up to 20 minutes) at a steam pressure of not more than 5 lbs. It would seem that the most satisfactory method would be suddenly to expose the fresh tissue to live steam. With the laboratory type of autoclave this is accomplished by having the instrument body full of steam, then suddenly opening the door introducing the plant tissue, and closing it up again at once. After all the air has been expelled the pressure is allowed to rise to 5 lbs. The pressure is then released suddenly, the tissue transferred to a press, and the sap expressed. The sap may then be prepared for use by some appropriate method. To make a fairly effective crude extract, 2 volumes of acetone may be added to the sap. After filtration the clear straw-colored filtrate is concentrated to the desired degree. If desired, this latter concentrate can then be purified by an appropriate method.

It has also been found that the active principle can be precipitated out from the fresh sap by the use of ammonium sulfate. This affords a very speedy and at the same time inexpensive method of obtaining potent extracts.

Blood sugar determinations were made by the Shaffer-Hartmann method.³

Results.

It has already been stated that the reaction of individual rabbits to injections of plant extracts is quite varied. Marked hypoglycemia may be manifested within 24 hours of the time of the injection or it may not be manifested for some days.

Some typical results illustrative of this type of phenomenon are shown below.

³ Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, xlv, 368.

Delayed or Prolonged Action.

Rabbit 1.—Apr. 18. A 1,940 gm. male rabbit was injected with 20 cc. of an extract prepared from green onion tops by first extracting frozen material with 5 volumes of 95 per cent acetone-denatured alcohol, filtering, and concentrating the filtrate in an air current to a light syrupy consistency. This syrup was saturated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate removed and extracted with 70 per cent alcohol. The mixture was then centrifuged, the clear liquid was added to 40 volumes of 95 per cent alcohol, and the precipitate resulting after adjustment of the reaction to neutrality was taken up in distilled water.

The extract used in this experiment would represent approximately 400 gm. of fresh onion material.

The blood sugar control was 0.108 per cent. 10 hours after the injection the blood sugar was 0.087 per cent. Subsequent readings were at 16 hours, 0.093 per cent; at 21 hours, 0.087 per cent; at $26\frac{1}{2}$ hours, 0.093 per cent; and at $32\frac{1}{2}$ hours, 0.118 per cent. The animal was then returned to the run apparently in a normal condition. On May 4, at 3 p.m. a blood sugar of 0.070 per cent was observed, while on May 5 at 9 a.m. the blood sugar was 0.112 per cent. The animal died suddenly on May 11. A sample of blood taken from the heart within 1 hour of death showed a sugar concentration of 0.020 per cent.

Apr. 18. An extract was prepared from 200 gm. of lawn grass as follows: 5 volumes of 95 per cent acetone-denatured alcohol were added to fresh cut grass. The mixture was boiled for 10 minutes on a water bath, a reflux condenser being attached. After filtration the clear filtrate was concentrated in a warm air current till the chlorophyll precipitated out. The fluid was then filtered and injected at once. The volume injected was 50 cc. The blood sugar control was 0.106 per cent. 15 minutes after the injection the animal became limp and lay on its side with its muscles completely relaxed. 10 hours later the animal was still limp; and the blood sugar was 0.143 per cent. $22\frac{1}{2}$ hours after the injection the animal had a convulsion and died immediately afterward. The blood sugar at death was 0.053 per cent.

Rabbit 2.—Apr. 20. A 2,205 gm. male rabbit was injected with 50 cc. of an extract of grass, made as in the previous instance and equivalent to 200 gm. of grass. The blood sugar control was 0.114 per cent. The animal became limp and listless 20 minutes following the injection and remained so for about 12 hours, after which time it recovered. $9\frac{1}{2}$ hours after the injection a blood sugar of 0.144 per cent was observed. The following blood sugar values were noted: after $18\frac{1}{2}$ hours, 0.103 per cent; after $22\frac{1}{2}$ hours, 0.096 per cent; and after $26\frac{1}{2}$ hours, 0.093 per cent. The animal was returned to the run apparently normal. On May 1, 11 days after the original injection, the animal was noted to be in a typical convulsion and quite helpless. A blood sample was taken and showed 0.045 per cent of sugar. 4 gm. of dextrose were injected and in 10 minutes the rabbit was sitting up

eagerly feeding. 24 hours later the blood sugar was 0.110 per cent, but the animal was very weak. Death resulted 2 nights later.

*Demonstration of a Hypoglycemia-Producing Principle in the
Blood Stream of Reacting Animals.*

Rabbit 3.—A male rabbit, weight 2,500 gm. which had been injected with 25 cc. of a partially purified onion extract on Apr. 26 without any hypoglycemia being observed on the 2 succeeding days was found in a listless state on May 12 and to have a blood sugar of 0.062 per cent. The animal was bled. 6 cc. of whole blood of this animal were injected at once into another rabbit (Rabbit 4, weight 640 gm.). The second animal was found dead the next morning, but was still quite warm. The blood sugar analysis showed 0.015 per cent of reducing sugar.

Rabbit 5.—On Apr. 30 a doe rabbit weighing 3,055 gm. was injected with 95 cc. of an extract of green onion tops made as follows: the fresh material after being frozen with CO₂ snow, was extracted with 3 volumes of hot water at 80°C. for 3 minutes, the mixture treated with 5 volumes of 95 per cent acetone-denatured alcohol, filtered, and the filtrate concentrated in a warm air current. The concentrate was finally filtered and injected. The equivalent of 320 gm. of onion tops in all was injected; the equivalent of 200 gm. at once and of 120 gm., 4 hours later. The blood sugar curve for 4 days was as follows:

Date.	Time.	Control.
		<i>per cent</i>
Apr. 30	3 p.m.	0.108
May 1	9 a.m.	0.112
	5 p.m.	0.108
	9 a.m.	0.080
May 2	5 p.m.	0.093
	9 a.m.	0.096
May 3	5 p.m.	0.103
	5 "	0.108
May 4		

The animal was returned to the run on May 4; and later, on May 16 at 10 a.m., it was found in a listless condition. Its weight was now 2,075 gm. It was bled at once. The blood sugar was 0.048 per cent. The blood was defibrinated and centrifuged. Three other rabbits (Rabbits 6, 7, and 9) were injected with the serum as follows:

Rabbit 6.—Male, weight 1,450 gm. May 16, 1 p.m. 14 cc. of serum injected; 9 cc. intravenously and 5 cc. subcutaneously. The blood sugar curve follows.

Date.	Time.	Blood sugar.
		<i>per cent</i>
May 16	6 p.m.	0.102
" 17	10 a.m.	0.080
	6 p.m.	0.099
May 18	9 a.m.	0.070
	4.30 p.m.	0.062

The rabbit died during the night. The blood taken post mortem was 0.015 per cent.

Rabbit 7.—Weight 445 gm. May 16, 2 p.m. 5 cc. of serum. May 17, 7 p.m. Listless. Bled from heart. Blood sugar 0.052 per cent. 1 gm. of glucose injected subcutaneously. Animal sat up in 5 minutes, but died at 11 p.m. May 18, 10 a.m. 3 cc. of this animal's blood serum injected into another small rabbit (Rabbit 8, weight 580 gm.). May 19, 8 a.m. Rabbit 8 found dead. Blood sugar post mortem was 0.019 per cent.

Rabbit 9.—May 16, 3 p.m. Rabbit 9, weight 583 gm. Injected with 20 cc. of fluid equal washings of 20 cc. of cells of defibrinated blood from which serum was separated for use in Rabbits 6 and 7 above. May 18, 3 p.m. Animal lying on side. Bled from carotid. Blood sugar, 0.024 per cent.

Rabbit 10.—A male rabbit of 1,971 gm. weight was injected on May 2 with 40 cc. of an extract made from 200 gm. of young green onion tops by CO₂ freezing, hot water extraction, and partial purification with alcohol. No ill effects were noted subsequently and blood samples taken twice daily for 3 following days did not show any marked hypoglycemia. The animal was returned to the run on May 5. It was found dead on the morning of May 12. The weight was now 1,621 gm. A blood sample taken post mortem showed 0.015 per cent sugar. 3 cc. of whole blood were injected into a small rabbit (Rabbit 11, weight 515 gm.) at 12 n. on May 12. On May 14 at 5 p.m. this animal was very weak and was bled from the carotid. The blood sugar was 0.053 per cent.

Rabbit 12.—On May 1 a male rabbit, weight 2,300 gm., was injected with 25 cc. of an extract made from 135 gm. of fresh lawn grass by the method referred to in the preceding experiment. The blood sugar curve was followed for 4 days, but no marked hypoglycemia was noted. The animal died during the night of May 7. On May 8 at 11 a.m. 5 cc. of whole blood taken from this animal post mortem were injected into a 700 gm. rabbit (Rabbit 13). May 9 at 12.30 p.m. Rabbit 13 had convulsions and died a few minutes later. The blood sugar at the time of death was 0.045 per cent.

Rabbit 14.—At 6 p.m., May 8, and again at 10.30 a.m., May 9, a male rabbit (weight 2,412 gm.) was injected with 16 cc. of a lawn grass extract made as follows. The fresh cut grass was placed in a canvas bag and thrust into a steaming autoclave. The steam pressure was allowed to rise to 5 lbs. after the displacement of air had been accomplished. The steam pressure was then suddenly released and the bag removed and placed in a

press. The sap which was pressed out was treated with 2 volumes of acetone, filtered, and the filtrate concentrated. The equivalent of 150 gm. of sap in all was injected.

The following blood sugar curve was obtained:

Date.	Time.	Control.
		<i>per cent</i>
May 8	6 p.m.	0.108
" 9	9 a.m.	0.087
	5 p.m.	0.096

May 10, 9 a.m. Animal listless, lying on side. Bled from heart. Blood sugar, 0.030 per cent. 10 a.m. 5 cc. of blood from Rabbit 14 were injected into a 750 gm. rabbit (Rabbit 15). May 11, 11 a.m. Rabbit 15 limp. Bled from heart. Died immediately after. Blood sugar, 0.042 per cent. 1 p.m. 4 cc. of blood from Rabbit 15 injected into a 500 gm. rabbit (Rabbit 16). May 12, 9 p.m. Animal weak. Blood sugar, 0.080 per cent. May 13, 8 a.m. Rabbit 16 found dead.

Rabbit 17.—On May 9 at 2 p.m. a female rabbit, weight 1,880 gm., was injected with 30 cc. of an extract made from 150 cc. of sap of the poplar catkin by the method described in the preceding experiment. The following blood sugar curve was obtained:

Date.	Time.	Control.	Remarks.
		<i>per cent</i>	
May 9	2 p.m.	0.100	
	5 "	0.100	
May 10	9 a.m.	0.087	
	5 p.m.	0.080	
May 11	9 a.m.	0.090	
	6 p.m.	0.108	
May 12	11 a.m.	0.020	Died suddenly.

7 cc. of blood from Rabbit 17 were injected into a 540 gm. rabbit (Rabbit 18) at 12 n., May 12. This latter animal (Rabbit 18) was found dead on the morning of May 13.

The above experiments demonstrate that there is a substance in certain plant extracts which will produce marked hypoglycemia in normal rabbits.

The fact that the onset of marked low blood sugar is so varied is at this time difficult of explanation. As the blood of an animal dying with hypoglycemia will produce the same symptoms in another rabbit injected with such blood, proof is afforded of the existence in the blood stream of a reacting animal of a specific principle.

Observations on a Totally Depancreatized Dog Which Lived 66 Days.

The complete results of the observations upon a depancreatized dog which had received in all, three injections of onion top extract, are shown below. The preliminary results of this experiment were reported in a previous communication.¹ As the animal became sugar-free and had a normal blood sugar level 3 days following the second injection of extract and as this condition persisted so long, the writer concluded that a small piece of pancreas must have remained in the animal and that this was functioning sufficiently to keep the blood sugar at a normal level. The diet was therefore at a later date greatly increased and it will be noted that a relatively great amount of lean meat was consumed without causing a comparable increase in sugar excretion. On April 27, the animal appeared to be reverting to a truly diabetic condition. A third injection was therefore made on May 2, and on May 12 the urine again became practically sugar-free. Death resulted on May 14. Professor Reveil of the Department of Anatomy of this University very kindly performed a postmortem examination. He was unable to find any pancreas on gross examination.

On Mar. 9 at 3 p.m. a 12 kilo, well nourished, mongrel dog was depancreatized. The following results were noted.

Date.	Time.	Blood sugar.	Urine volume.	Sugar in urine.	Food eaten and remarks.
		<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	
Mar. 9	3 p.m.				Depancreatized.
" 10	9 a.m.		210	6.15	
	11 "				3 oz. lean meat.
Mar. 11	12 n.		320	1.57	
" 12	9 a.m.		216	3.82	
	10 "	0.160			8 oz. lean meat.
	4.30 p.m.	0.200			
Mar. 13	9 a.m.	0.160	300	3.18	
	4.30 p.m.	0.190			
Mar. 14	9 a.m.		235	3.16	
	10 "	0.161			8 oz. lean meat.
	3.30 p.m.				100 cc. of onion extract = 500 gm. of onion tops.

Date.	Time.	Blood sugar.	Urine vol- ume.	Sugar in urine.	Food eaten and remarks.
		<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	
Mar. 14	6.30 p.m.	0.224			
	11.30 "	0.190			
Mar. 15	9 a.m.		415	5.25	
	10 "	0.106			
	11 "		105	0.67	Container not washed after last sample was taken.
	4.30 p.m.	0.187	105	1.05	
Mar. 16	9 a.m.		320	5.95	
	10 "	0.168			8 oz. lean meat.
	5 p.m.	0.190			
Mar. 17	8.30 a.m.		480	4.84	
	1 p.m.	0.190			8 oz. lean meat.
	1.30 "		180	5.74	170 cc. of onion extract = 700 gm. onion tops.
	4 "				Vomited, but ate vomit again later.
	4.30 "		100	8.45	
Mar. 18	1 a.m.		220	7.10	
	3 "	0.180			
	10 "				8 oz. lean meat.
	11 "	0.148			
	12.30 p.m.		190	1.49	
	5 "	0.106			
	9.30 "	0.093			
Mar. 19	1 a.m.	0.118			
	9 "		108	0.20	
	10.30 "	0.090			
	11 "				8 oz. lean meat.
	5 p.m.		172	0.17	
	6 "	0.148			
Mar. 20	9.30 a.m.	0.180	208	0.66	
	6 p.m.	0.158			
	11 "		180	1.63	
Mar. 21	10 a.m.	0.090			Abscess opened. Fed 8 oz. meat. Did not eat till some hours later.
	1.30 p.m.		146	0.70	
	5 "	0.099			
Mar. 22	9 a.m.		188	0	
	10 "	0.108			8 oz. lean meat.

Date.	Time.	Blood sugar.	Urine volume.	Sugar in urine.	Food eaten and remarks.
		<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	
Mar. 23	10 a.m. 12 n.		435	0	8 oz. lean meat. Large abscess opened. ½ pint of pus removed.
Mar. 24	9 a.m. 10 "		482	0	8 oz. lean meat.
Mar. 25	10 "		246	0	8 " " "
" 26	10 "		340	0	12 " " "
" 27	10 "		272	0	20 " " "
" 28	10 "		284	0	32 " " "
" 29	10 " 5 p.m.		390 310	0 0.93	40 " " "
Mar. 30	10 a.m.		265	0	40 oz. lean meat.
" 31	10 "		445	0.97	32 " " "
Apr. 1	10 "		500	0.85	40 " " "
" 2	10 "		700	0.87	40 " " "
" 3	10 "		550	1.32	48 " " "
" 4	10 "		600	1.82	48 " " "
" 5	10 "		615	0.24	48 " " "
" 6	10 " 3 p.m.		600	1.05	48 " " "
Apr. 7	10 a.m.	0.180 0.080	412	0.78	48oz. lean meat. Weight, 8.45 kilos.
Apr. 8	10 "		670	0.78	40 oz. lean meat
" 9	10 "		640	1.00	48 " " "
" 10	10 "		640	1.40	48 " " "
" 11	10 "		530	0.75	48 " " "
" 12	10 "		675	1.88	48 " " "
" 13	10 "		655	0.46	48 " kitchen scraps.
" 14	10 "		810	3.64	40 " " "
" 15	10 "		605	3.35	Not fed.
" 16	10 "		340	0	24 oz. kitchen scraps.
" 17	10 "		1,310	6.94	24 " " "
" 18	10 "		465	2.62	37 " " "
" 19	10 "		785	3.73	50 " " "
" 20	10 "		1,615	4.42	44 " " "
" 21	10 "		1,790	4.75	56 " " "
" 22	10 "		1,980	4.78	Not fed.
" 23	10 "		575	1.40	48 oz. kitchen scraps.
" 24	10 "		980	3.20	Not fed.
" 25	10 "		220	1.70	" " "
" 26	10 "		196	0	" " "
" 27	10 "		185	0.74	8 oz. lean meat.

Date.	Time.	Blood sugar.	Urine vol- ume.	Sugar in urine.	Food eaten and remarks.
		<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	
Apr. 28	10 a.m.		223	2.54	8 oz. lean meat.
" 29	10 "				8 " " "
" 30	10 "		410	1.60	8 " " "
May 1	10 "		230	4.80	8 " " "
" 2	8.30 "		445	4.10	
	10 "				8 oz. lean meat.
	2 15 p.m.		215	6.88	
	3 "	0.240			Weight, 7.100 kilos.
	3.30 "				Injected 50 cc. of onion extract = 300 gm. onion tops.
May 3	8.30 a.m.		215	8.26	
	10 "				8 oz. lean meat.
	1 p.m.		80	8.96	
	3 "	0.168			
May 4	8.30 a.m.		130	7.20	
	10 "				8 oz. lean meat.
	12 n.		90	2.82	
	3.30 p.m.		50	3.88	
May 5	8.30 a.m.		184	2.45	
	10 "				8 oz. lean meat.
	5 p.m.		190	3.87	
May 6	10 a.m.				8 oz. lean meat.
	12 n.		170	1.51	
	7 p.m.		80	3.96	
May 7	9 a.m.		90	1.40	
	10 "				8 oz. lean meat.
May 8	9 "		300	3.70	
	10 "				8 oz. lean meat.
May 9	8.30 "		236	2.65	
	10 "				8 oz. lean meat.
May 10	8.30 "		325	1.16	
	10 "				8 oz. lean meat.
May 11	8.30 "		180	2.39	
	10 "				8 oz. lean meat.
May 12	8.30 "		274	Mere trace.	
	10 "				8 oz. lean meat.
May 13	10 "				8 " " "
	11 "		130	Mere trace.	
May 14	9 "		205	" "	
	p.m.				Died during night.

An active principle was shown to be present in the blood of this animal after death as marked hypoglycemia was produced in a series of rabbits by using the blood serum prepared from heart blood taken post mortem.

DISCUSSION.

The fact that intensive action of the principle (blood sugar below 0.05 per cent) may be manifested at such varying intervals of time following the initial injection of the plant extract as clearly illustrated in the results shown above is very difficult of satisfactory explanation. As a relatively small volume of blood or serum taken from a reacting animal either before death or even some hours after death produces with such uniformity characteristic intensive hypoglycemia when injected into another animal, it might appear that the marked hypoglycemia of the animal receiving the plant extract was due directly to the presence in the circulation of an excessive amount of some principle irrespective of whether the injection of plant extract has been made 1 day previous or even weeks previous. That the hypoglycemia in the animal receiving the blood or serum of the reacting animal develops within a comparatively short space of time (1 to 3 days) is also a noteworthy fact.

The blood serum of an animal in hypoglycemia as a result of the inoculation with serum obtained from a reacting animal which originally received plant extract, will also produce marked hypoglycemia in a third animal. This is suggestive that an actual increase in the amount of active principle has been effected.⁴

⁴ *Addenda*.—The blood of rabbits, which were maintained in a condition of hypoglycemia as a result of various causes, has produced hypoglycemia when injected into other normal rabbits. The blood of these later inoculated animals has likewise produced a similar condition in other rabbits. The blood of the third animal in such a series has produced profound hypoglycemia in a fourth animal, the blood of the fourth in a fifth and so on, apparently without limit.

Animal passage of a hypoglycemia-producing principle (which has been found to be thermostable) is possible, therefore, to an indefinite extent.

The relationship, if there is any, between the causative agent of hypoglycemia in the first animal of a blood passage series and the hypoglycemia-producing principle, present in the blood of a reacting animal is yet to be established.

Another interesting observation is the fact that injected rabbits, though eating ravenously in the presence of an abundance of food, continue to lose weight and when they finally die with a low blood sugar, may be very emaciated. These observations are suggestive of an intensive metabolism, of protein in particular, but as yet no actual determinations of metabolic rates or nitrogen elimination have been made.

A great many experimental animals which manifested only a mild hypoglycemia or in some instances practically no hypoglycemia within the 3 days following the injection have been found dead in the run many days later, usually in an emaciated condition. Postmortem blood analysis invariably showed a very low blood sugar, but as this might have been due to normal postmortem glycolysis these results have been discarded. They are, however, highly suggestive. Since it has been found, however, that injection of the blood from a reacting animal even though taken after death will produce hypoglycemia of a marked degree when injected into another animal, a method is afforded of determining with a fair degree of certainty whether or not an injected animal which has died unobserved, had a low blood sugar at the time of death.

Unfortunately, due to the fact that the writer himself had lost faith in the experiment, the observations on the totally depancreatized dog which lived for 66 days are not as complete as

A series of animal passages of a hypoglycemia-producing principle has been made from: (1) rabbits inoculated with certain plant extracts, (2) insulinated rabbits, (3) rabbits in hypoglycemia following the injection of guanidine sulfate, and (4) rabbits developing spontaneous hypoglycemia (coccidial origin).

A remarkable parallelism between the effect of guanidine sulfate, of passage serum, and certain plant extracts has been observed. All three reagents produce hypoglycemia in normal rabbits. The condition of low blood sugar does not develop until many hours after the injection. Convulsions usually occur when a very low level of blood sugar is reached. The symptoms are as a rule temporarily relieved by glucose administration, but they continue to recur and death has ultimately resulted in spite of glucose administration.

The writer is convinced that insulin is a fairly simple compound, non-protein in nature. It is possible that it may be a guanidine compound.

Further detailed results obtained in this investigation are about to be published.

could be desired. The fact, however, that for a period of many days following the injection of an onion extract the urine was sugar-free is indeed remarkable. It would appear that whatever this plant principle may be, that it acts in a somewhat different manner from insulin. Clinical trial of this substance has been postponed, until more definite scientific facts as to the nature of the physiological response here met with, are determined.

SUMMARY.

1. Further observations on the effects of plant extracts on blood sugar are recorded.
2. Various methods of obtaining potent extracts from plant tissues are described.
3. The characteristic sign of the activity of glucokinin "low blood sugar" may be manifested from 1 day to several weeks after the injection has been made.
4. A totally depancreatized dog was kept alive 66 days. The animal received in all, three injections of glucokinin (onion extract).
5. Lawn grass as a source of glucokinin has been found to give satisfactory results.
6. Animal passage of a hypoglycemia-producing principle is possible.

I wish to express my thanks to the Council of the College of Physicians and Surgeons of the Province of Alberta for supplying funds which made this investigation possible.

AGE AND CHEMICAL DEVELOPMENT IN MAMMALS.*

By C. R. MOULTON.

(From the Bureau of Nutrition, Institute of American Meat Packers, Chicago.)

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It has long been recognized that the most striking change in the composition of mammals with increased age and development is the increase in the fat content. Where fattening did not intervene to obscure the changes in the other constituents it was recognized that age and species determined largely the composition of the animal. Thus, von Bezold¹ in 1857 concluded as a result of analytical investigations with a large number of species of animals that: Every individual animal possesses a normal water, organic matter, and salt content which is typical of its species and age, and which is approximately constant (the higher vertebrates) or varies between wider or narrower limits (the mollusks). He further described the changes occurring in the composition of mammals, birds, and amphibians as: (1) A decrease in the content of water and fluid constituents beginning with the development of the embryo and continuing up to the height of early growth. (2) An increase in the solid organic material which occurs at the greatest rate during the first period of growth after birth. (3) The ash content increases continuously and rapidly during the first period of autonomous life and proceeds more slowly later. No determinations were made of the fat content of the animals.

The attention of others was attracted to this same question and Pfeiffer,² in 1887 when discussing his results and those of others in connection with the known effect of fat deposition, quoted Hösslin as follows:

* Presented before the Division of Biological Chemistry, American Chemical Society, New Haven, Conn., April, 1923.

A preliminary report on part of this material was given before the University of Missouri section of the American Chemical Society, December, 1922.

¹ von Bezold, A., *Z. wissenschaft. Zool.*, 1857, viii, 487.

² Pfeiffer, L., *Z. Biol.*, 1887, xxiii, 340.

"It has been known for a long time that fat deposition in the tissues exerts a great influence on the water content of the same; the organism of the fattened animal contains less water, the richer it is in fat. The question is according to Voit in part one of the replacement of water by fat but chiefly one of deposition of water-free fat in the tissues so that after the removal of the latter the organism shows a nearly normal water content."

More recently (1911) Inaba³ investigated the differences in the composition of animals and concluded that morphologically similar animals have a similar composition of body substance. He pointed out that warm blooded animals are richer in fat than cold blooded animals and that of the former, those which run around and are not sheltered by the mother at birth (guinea pig) are richest in fat. He further showed that the water content of young individuals is richer than is the case with older animals and the younger, the higher the water content.

Quite recently (1922) Murray⁴ made a study of the composition of our agricultural animals on the fat-free basis. He shows that, for the animals used in his calculations, the chemical composition of the non-fatty matter is practically the same, that it is not affected by the condition or degree of fatness of the animal and only to a limited extent by age.

In contrast to the conclusions just cited are those of the writer which were based on the fat-free composition of the ox and man from the early embryonic stage on through maturity.⁵ It was shown that the water content of such mammals decreases very rapidly from conception to birth, and then less rapidly until a practically constant concentration is reached. For the ox this is between the ages of 5 and 10 months. The point at which the concentration of water, proteins, and salts becomes comparatively constant in the fat-free cell is named the point of chemical maturity of the cell. The words "chemical maturity" in this paper refer to this point and carry with them no implications of too general a nature.

Fig. 1 shows graphically the results on which the above statement is based.

The present communication will test these last conclusions further and present what appear to be general laws of chemical

³ Inaba, R., *Arch. Physiol.*, 1911, 1.

⁴ Murray, J. A., *J. Agric. Sc.*, 1922, xii, 103.

⁵ Moulton, C. R., Trowbridge, P. F., and Haigh, L. D., *Missouri Agric. Exp. Station Research Bull.* 55, 1922, 21.

development for mammals. The extensive data for cattle and swine collected by the writer and presented in detail elsewhere⁶ will be presented here only in graphical form. The data for other mammals are given in Tables I to III.

TABLE I.
Percentage Composition of Man on a Fat-Free Basis.

Age.	Investigator.	No. of individuals.	Water.	Nitrogen.	Ash.
Fetus.					
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
35 days.	Fehling. ⁷	1	97.54	0.39	0.001
2.5 mos.	Michel. ⁸	1	93.82	0.69	
3 " "	Fehling. ⁷	2	91.84	0.81	1.005
3-4 " "	Michel. ⁸	1	89.95	1.1	1.73
4 " "	Fehling. ⁷	2	92.46	0.92	1.23
4.5 " "	"	5	90.38	1.11	1.62
5 " "	"	3	87.43	1.26	2.40
5 " "	Michel. ⁸	1	87.81	1.32	1.95
5 " "	"	1	86.73	1.39	2.49
6 " "	"	1	85.03	1.64	2.51
6 " "	Fehling. ⁷	4	86.00	1.78	2.72
7 " "	"	1	84.97	1.71	2.89
7 " "	Michel. ⁸	1	84.74	1.56	2.49
Full term.	Fehling. ⁷	1	81.52	2.08	2.81
Extrauterine.					
New-born	Camerer and Söldner. ⁹	6	81.87	2.13	3.08
" "	Klose. ¹⁰	1	80.2	2.32	3.52
3 mos.	Sommerfeld.	1	80.73	2.61	3.14
4 " "	Steinitz and Weigert. ¹¹	1	77.75	2.97	3.94
33 yrs.	Moleschott. ¹²	1	69.33	3.3	9.44?

⁶ Armsby, H. P., and Moulton, C. R., The animal as a converter of matter and energy, American Chemical Society Monograph, New York, 1923, in press.

⁷ Fehling, H., *Arch. Gynäk.*, 1877, xi, 523.

⁸ Michel, C., *Compt. rend. Soc. biol.*, 1899, li, 422.

⁹ Camerer, W., *Z. Biol.*, 1900, xxxix, 173; xl, 529; 1902, xliii, 1. Söldner, *Z. Biol.*, 1903, xlv, 61.

¹⁰ Klose, E., *Jahrb. Kinderh.*, 1900, xxx, 253.

¹¹ Steinitz, F., and Weigert, R., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 206.

¹² Moleschott, J., *Physiologie der Nahrungsmittel*, Giessen, 2nd edition, 1859, 224.

TABLE II.
Percentage Composition of Rodents on a Fat-Free Basis.

Age.	Investigator.	No. of individuals.	Water.	Nitrogen.	Ash.
Guinea pig.					
Embryo.	Inaba. ³	1	per cent 88.29	per cent 1.38	per cent 1.57
New-born.	"	1	77.79	2.69	3.76
2-3 mos.	Weigert. ¹³	1	76.41	3.04	4.39
Grown.	"	1	74.54	3.54	4.83
Rabbit.					
12 day embryo.	Fehling. ⁷	2	91.45		
18 " "	"	2	88.17	1.38	1.43
21 " "	"	6	87.42	1.43	1.80
24 " "	"	6	86.01	1.70	2.72
27 " "	"	2	83.44	1.93	2.63
At birth.	"	2	83.24	2.16	2.94
" "	Reeb. ¹⁴	16	84.22	2.00	2.05
Embryo.	Inaba. ³	1	88.32	1.45	1.82
Grown.	Rubner-Inaba.	1	72.82	3.11	4.59
"	Pfeiffer. ²	1	69.5		
"	"	1	71.6		
Rat.					
At birth.	Zuntz. ¹⁵	281	88.92	1.53	1.50
" "	Hatai. ¹⁶	1	88.80		1.53
7 days.	"	1	85.99		2.05
15 "	"	1	81.54		2.46
22 "	"	1	79.15		3.25
28 "	"	1	78.64		3.39
35 "	"	1	76.41		3.35
42 "	"	1	75.68		3.82
294 "	"	1	69.25		3.92
Mature.	Inaba. ³	1	74.59	3.33	3.43
Mouse.					
Embryo.	von Bezold. ¹	5	87.16		1.14
New-born.	Inaba. ³		85.97	1.76	1.65
Mature.	"		78.33	3.24	3.52
"	Bohtlingk-Inaba.		75.41	3.39	3.41
"	Rubner-Inaba.		75.18	2.72	3.94

¹³ Weigert, R., *Jahrb. Kinderh.*, 1905, lxi, 178.

¹⁴ Reeb, M., *Beitr. Geburtsh. u. Gynäk.*, 1904-05, ix, 395.

¹⁵ Zuntz, L., *Arch. Gynäk.*, 1918-19, cx, 244.

¹⁶ Hatai, S., *Am. J. Anat.*, 1917, xxi, 23.

TABLE III.

Percentage Composition of the Dog and Cat on a Fat-Free Basis.

Age.	Investigator.	No. of individuals.	Water.	Nitrogen.	Ash.
Dog.					
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Birth.	Thomas. ¹⁷	2	81.17	2.28	2.24
"	Reeb. ¹⁴	2	82.00	2.15	1.94
9 days.	Thomas. ¹⁷	1	81.04	2.35	2.33
20 "	"	1	78.93	2.55	2.69
59 "	"	1	78.63	2.64	2.87
100 "	"	1	74.72	3.09	3.90
3.5 mos.	Weigert. ¹³	2	76.45	3.67	4.14
4.5 "	"	3	70.98	3.66	5.74
5.5 "	"	1	74.27	3.53	4.94
5.5 "	"	1	80.44?	3.35	6.34?
Grown.	"	1	69.76		
"	Pfeiffer. ²	3	69.2		
Cat.					
Birth.	Thomas. ¹⁷	2	82.18	2.29	2.61
9 days.	"	1	82.96	2.17	2.26
22 "	"	1	79.45	2.58	2.64
103 "	"	1	72.40	3.49	3.53

Cattle.

The effect of age on the composition of cattle is shown in Figs. 1 and 2. In Fig. 1 the results of Fehling on human embryos are shown by the squares. Fig. 2 presents the water data for cattle from four groups of investigators. The human data are omitted. It is evident that the exclusion of the human data and the inclusion of other than the data of Moulton and coworkers does not affect the curve. From Fig. 1 it is seen that cattle develop in the concentration of protein (nitrogen) and ash very rapidly from early intrauterine life until the age of 5 months is reached. At about that time the change becomes suddenly very slow and in the case of nitrogen no further change is seen. The ox at birth contains 76 per cent water, and man about 82 per cent. The curve for water shows just the reverse phenomenon; *i.e.*, a rapid decrease in concentration on the fat-free basis and then a practically constant percentage after 5 months.

¹⁷ Thomas, K., *Arch. Physiol.*, 1911, 9.

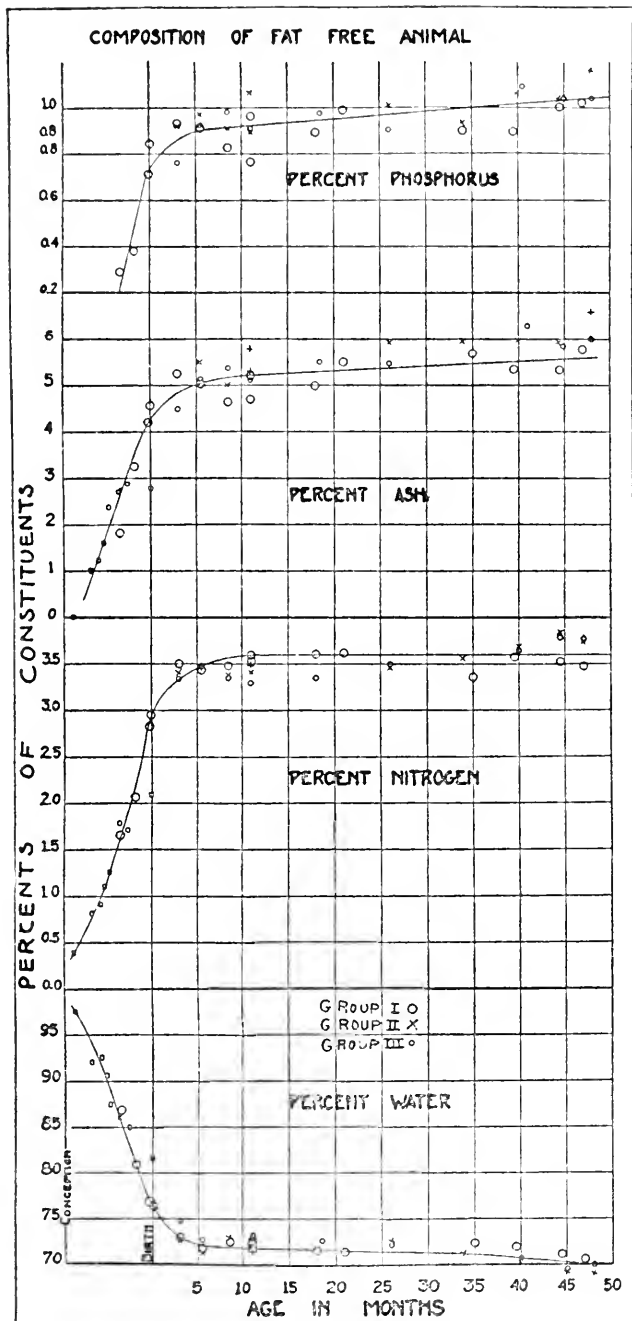


FIG. 1. Effect of age on the composition of cattle. Data of Moulton and coworkers and data on human embryos.

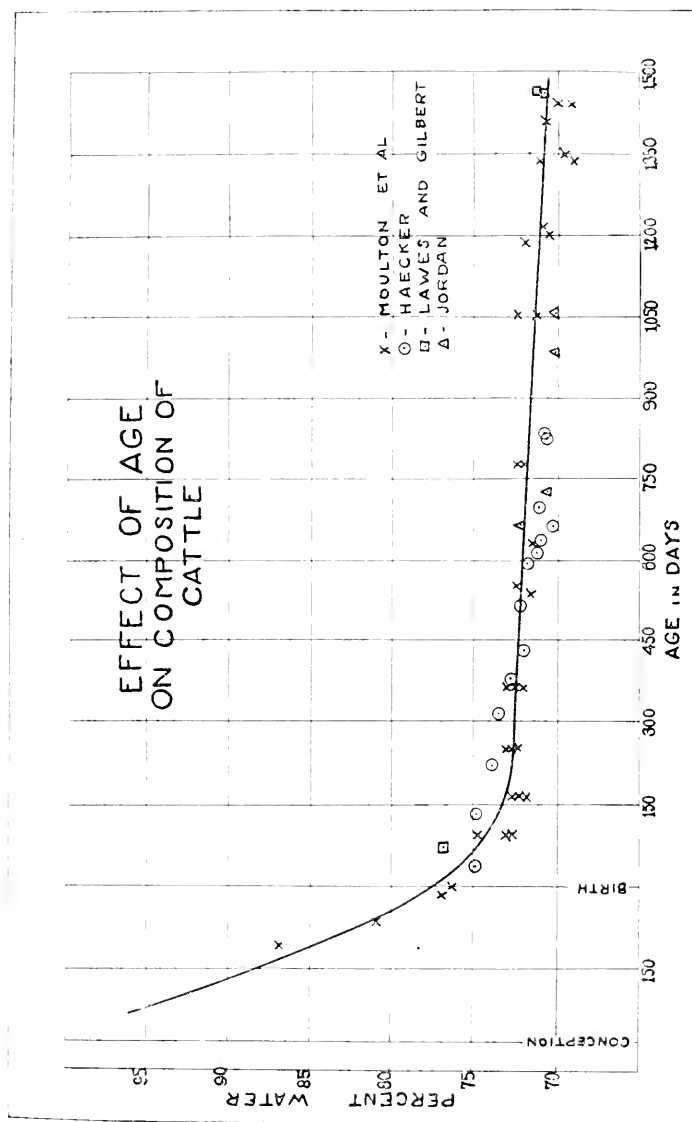


FIG. 2. Effect of age on the composition of cattle. Data of Moulton and coworkers; Haecker; Lawes and Gilbert; and Jordan.

Guinea Pig.

Fig. 3 shows the data for the guinea pig. Quite similar changes are shown although the curves do not flatten completely. This may be the result of insufficient data. The point of change is about the 50th day. The water content at birth is about 78 per cent and the nitrogen and ash percentages are very similar to those for cattle.

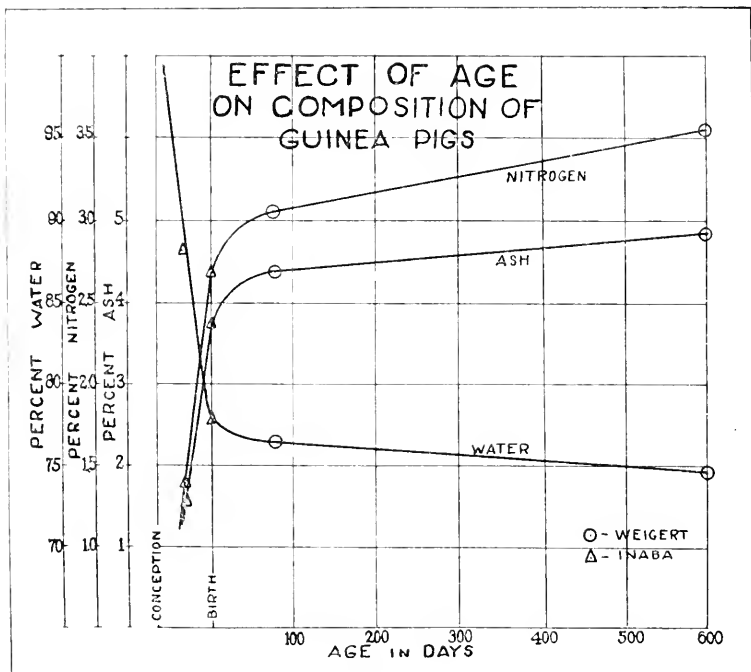


FIG. 3. Effect of age on the composition of guinea pigs.

Man.

The data for man shown in Fig. 4 are quite numerous up to 3 to 4 months. There is but one result after that age, but being for an adult it serves the purpose of fixing the direction of the curve. The curve for ash is located near the center of the figure in order to avoid confusion. The curves are similar to those shown in Figs. 1, 2, and 3. If one may venture to predict the

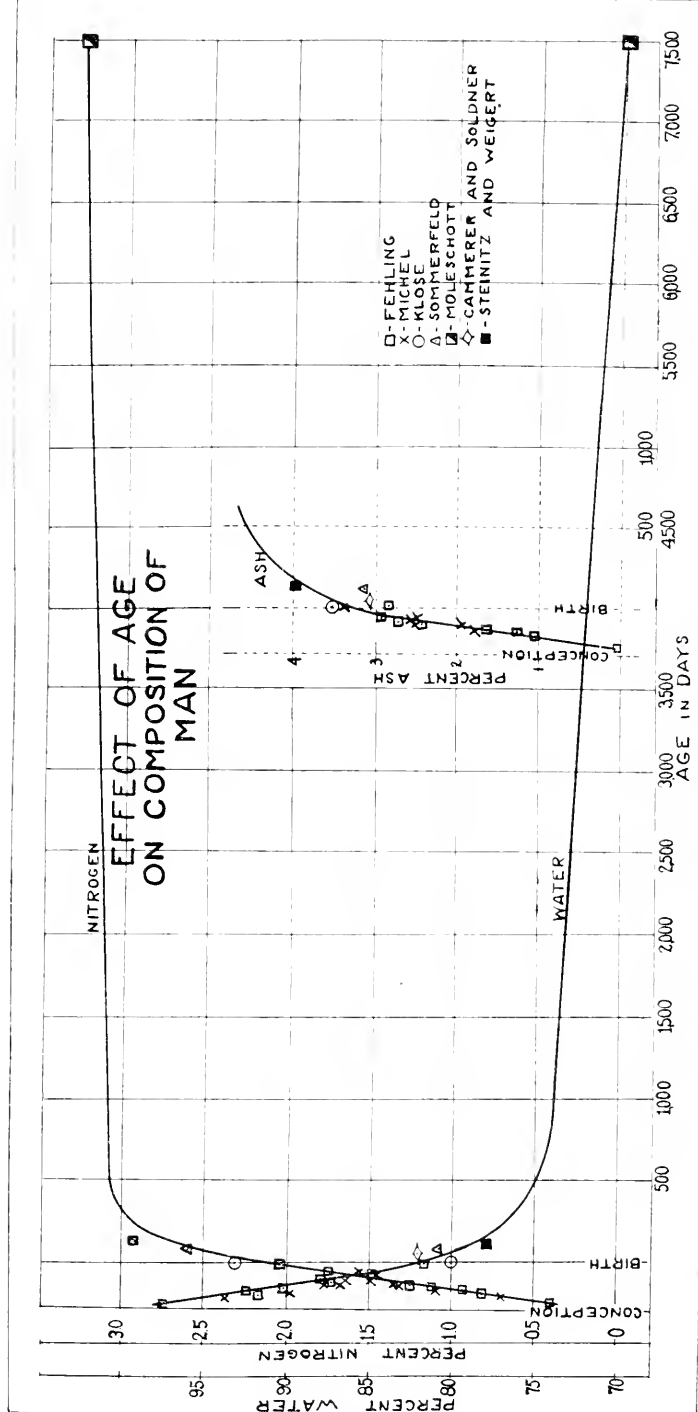


Fig. 4. Effect of age on the composition of man.

time of chemical maturity from the scant figures after birth, it would be placed between 500 and 1,000 days. A greater part of the chemical development is shown to occur after birth than is the case with the ox and the guinea pig.

Swine.

Figs. 5 and 6 give the data for swine. The results are more scattered than those shown so far and the time of maturity is

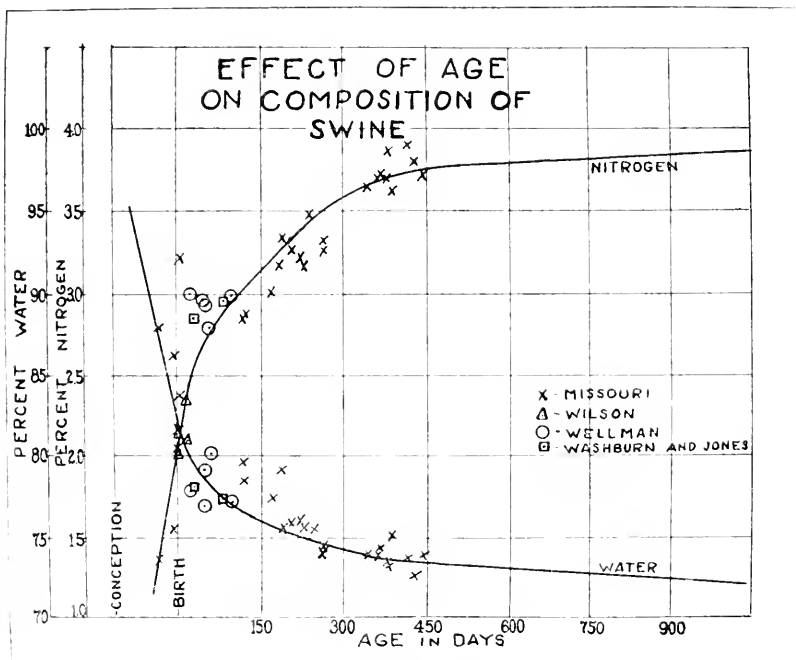


FIG. 5. Effect of age on the composition of swine. Water and nitrogen content.

not easily fixed, but in general the curves are similar to those in Figs. 1 to 4. The data of Swanson on pigs are not used for reasons set forth in another publication.⁶ Chemical maturity is reached at 150 to 300 days, but the point might be changed by more data. Swine at birth contain about 82 per cent of water. Apparently the nitrogen content is higher and the ash lower than for man.

Dog.

The striking thing to be seen in Fig. 7 is that apparently maturity for dogs is reached more slowly. The slope of the rising part of the curve depends on the age scale, but the relative amount of development after birth is not affected by this scale. The dog shows relatively more chemical development after birth than do the animals so far discussed. At birth the dog

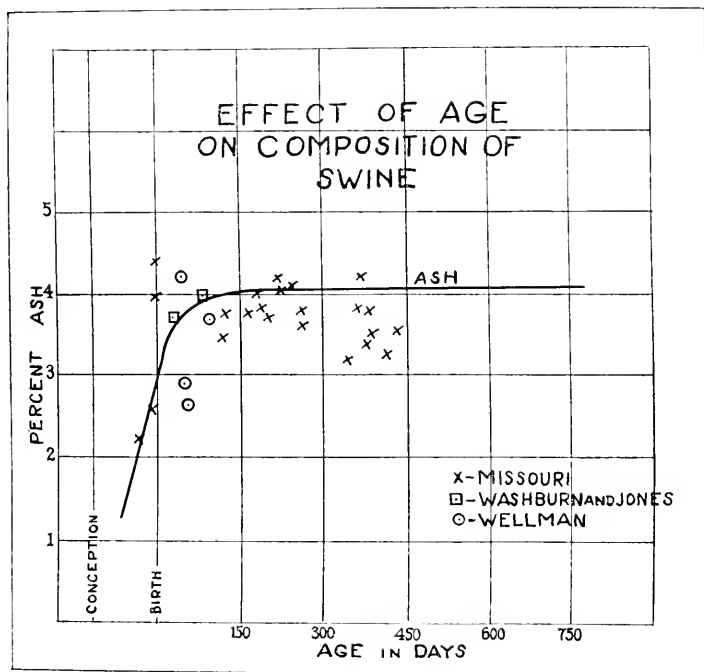


FIG. 6. Effect of age on the composition of swine. Ash content.

contains about 82 per cent water, 2.3 per cent nitrogen, and about 2.2 per cent ash. Chemical maturity is reached near the age of 200 days.

Cat.

Data for the cat are few. Fig. 8 shows curves apparently similar to those for the dog. It is difficult to predict chemical maturity, but it would appear to be near 100 days.

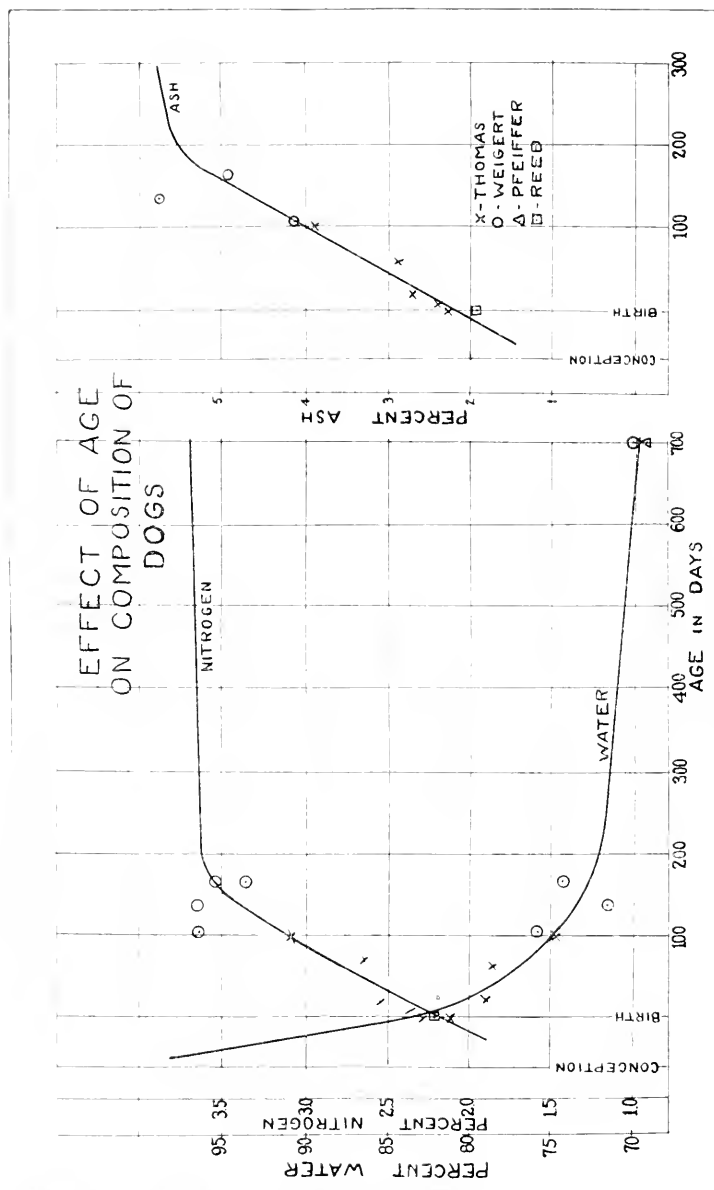


FIG. 7. Effect of age on the composition of dogs.

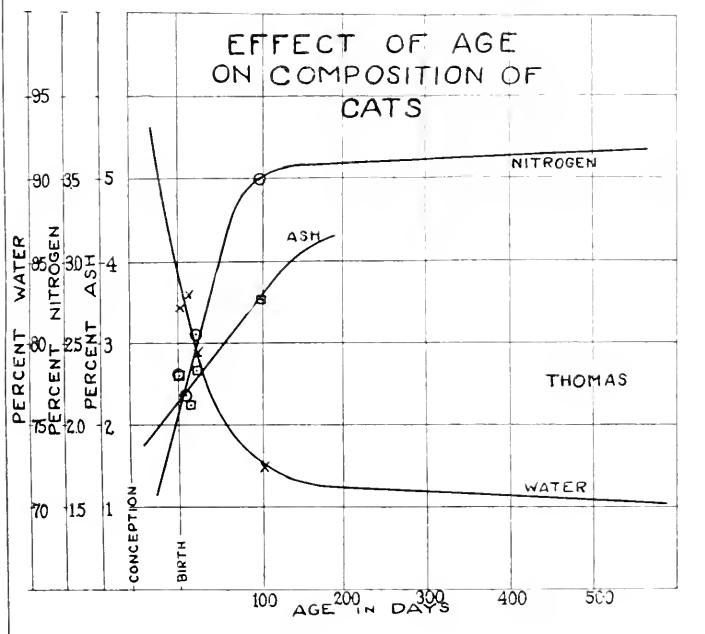


FIG. 8. Effect of age on the composition of cats.

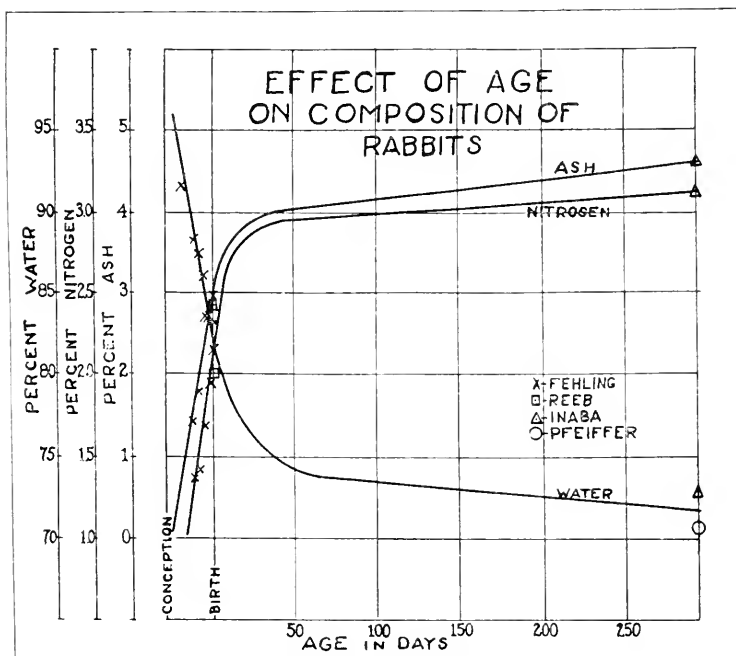


FIG. 9. Effect of age on the composition of rabbits

Rabbit.

Fig. 9 gives the data for rabbits. The water content at birth is 83 to 84 per cent. The age of chemical maturity cannot be predicted because of a lack of figures between birth and maturity. In general the curves are similar to those for the animals just preceding.

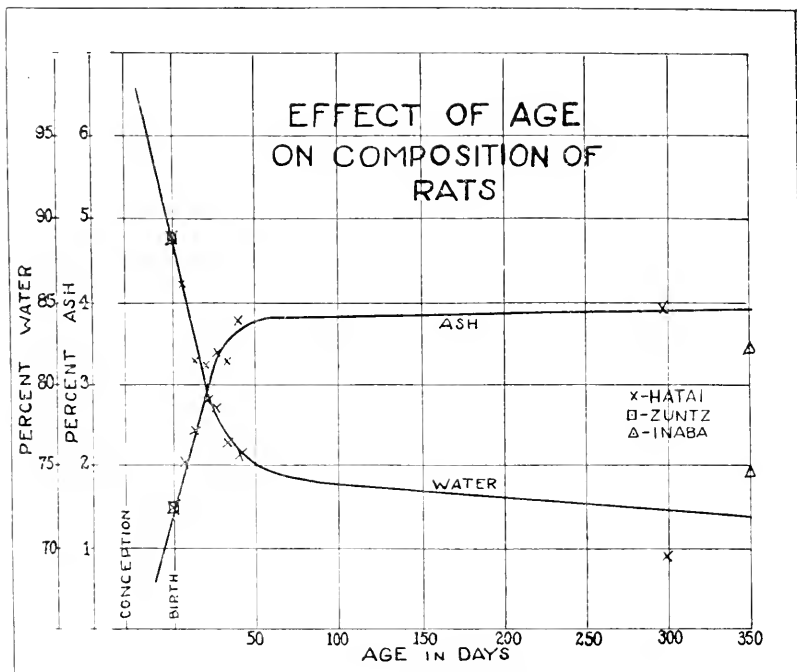


FIG. 10. Effect of age on the composition of rats.

Rat.

The data for the rat are given in Fig. 10. No data for nitrogen are given since these are almost entirely lacking in the literature. Relatively more development after birth is shown by the rat, and it is born quite immature. It contains 88 per cent water and only 1.5 per cent ash at birth. Chemical maturity is reached at the age of 50 days.

Mouse.

The data for the mouse given in Fig. 11 are not sufficient for fixing the curves. They have, however, been drawn similar to those for the rat. At birth the mouse contains 86 to 87 per cent water and about 1.7 per cent ash.

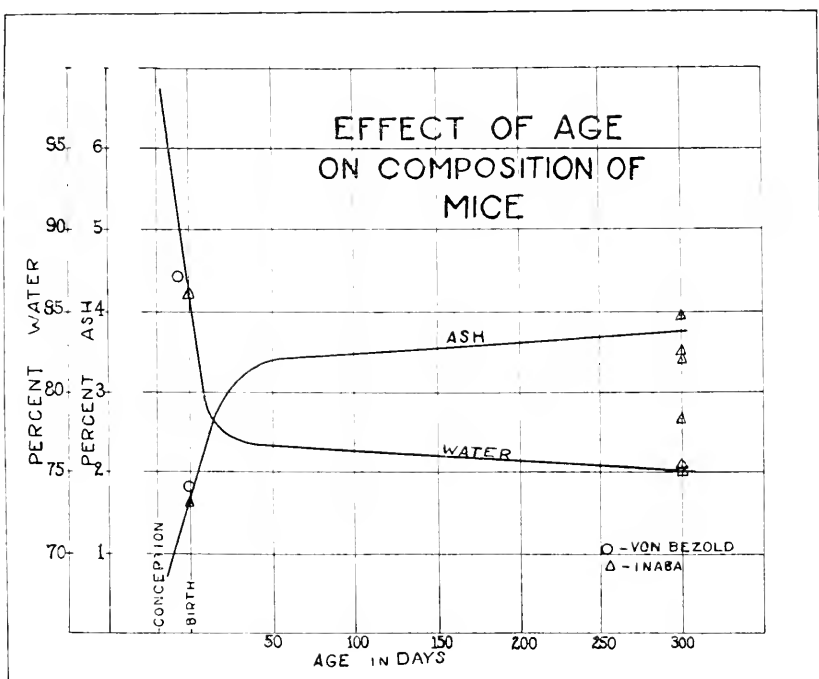


FIG. 11. Effect of age on the composition of mice.

DISCUSSION.

All the mammals for which data have been presented show a rapid decrease in the concentration of water on the fat-free basis from the earliest days of life until chemical maturity is reached. On the other hand, the protein (nitrogen) and ash concentration increases in the same measure. Thereafter, but little change is shown.

The mammals vary in the relative amount of chemical development shown at birth. Both guinea pigs and cattle show relatively great development at birth (Group 1).

Man, the pig, the dog, and the cat all have similar composition at birth (Group 2). The water content is higher than for the ox and guinea pig and relatively less chemical development has occurred. The animals of Group 1 quickly get on their feet at birth and are well developed physically. Group 2 shows somewhat less physical development at birth.

The rabbit may be less developed chemically at birth than the animals in Group 2. More data would be needed before this could be stated as a law. It may occupy a position between Group 2 and Group 3 (the two rodents).

TABLE IV.

Composition of Normal and Abnormal Infants on a Fat-Free Basis.

Age.	Condition.	Water.	Protein.	Ash.	Investigator.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Birth.	Normal.	82.00	14.00	3.00	
3 mos.	"	80.73	16.33	3.14	Sommerfeld. ¹⁸
24 days.	Edematous.	87.5	9.99	2.83	Klose. ¹⁹
3 mos.	Gastrointestinal disorders.	81.08	14.69	2.77	Steinitz. ²⁰
3.5 "		83.81	13.56	3.26	"
3.75 "		81.52	16.00	3.41	"

The rat and the mouse (Group 3) are very immature at birth and are most dependent upon their mother. The greater part of their chemical development occurs after birth and they have the highest water content at birth.

The relative fatness of animals of the same species does not effect the composition when calculated to the fat-free basis. This is especially well shown by the curves for cattle since the animals were of all degrees of fatness and still approximate the curves very closely.

It would apparently follow from the preceding paragraph that the plane of nutrition would not affect the composition of animals.

¹⁸ Sommerfeld, P., *Arch. Kinderh.*, 1900, xxx, 253.

¹⁹ Klose, E., *Jahrb. Kinderh.*, 1914, lxxx, 154.

²⁰ Steinitz, F., *Jahrb. Kinderh.*, 1904, lix, 447.

This is true unless the conditions have been such as to retard development of the individual. Some indication of this effect is shown by atrophic infants and infants suffering from gastrointestinal disorders from birth and consequently badly nourished. The data in Table IV show a noticeably higher water content for the abnormal infants, the difference being from 1 or 2 to 7 per cent higher. Marked underdevelopment at birth is also accompanied by high water content as shown by Jersey Calf 85A reported by Haigh, Moulton, and Trowbridge.²¹ This calf weighing only 30 pounds at birth, was the offspring of a mother fed an abnormally low mineral ration and at birth it showed an undeveloped lower jaw and the absence of eyes. On the fat-free basis its water content was 78.2 per cent, while normal calves contain 76.2 per cent water.

Relation between Chemical Development and the Life Cycle.

In Table V are presented the ages of the various mammals studied at different times in their life cycles. The ages shown are those based on conception as the point of reference. The approximate length of life is based largely on figures in the 9th and 11th editions of the Encyclopedia Britannica and in Metchnikoff's "The prolongation of life." They are probably as accurate as can be found. It is seen that with some of the animals the length of the gestation period and the composition at birth are correlated. Thus, cattle with a long gestation period are quite mature at birth. The cat and dog have shorter gestation periods and are less mature at birth, and the rat and mouse, with the shortest periods, are least mature. Man, the pig, and especially the guinea pig do not show this relation. There is still less correlation with the length of life. About as good correlation is shown between the age at the maximum of the third growth cycle and the composition at birth. The figures for the third growth cycle are those of Brody and Ragsdale.²²

If the age at which chemical maturity is reached is divided by the length of life—both expressed in days of conceptional age

²¹ Haigh, L. D., Moulton, C. R., and Trowbridge, P. F., *Missouri Agric. Exp. Station Research Bull.* 28, 1920.

²² Brody, S., and Ragsdale, A. C., *J. Gen. Physiol.*, 1922-23, v, 205.

—and the results expressed as percentages, a fairly constant figure is obtained. Had the length of life of the animals been more carefully determined or had the age at chemical maturity been more accurately fixed, this percentage might be more constant. It is sufficiently constant to permit the formulation of the following tentative law: That mammals reach chemical maturity at different ages, but these ages are a fairly constant relative part of the total life cycle.

TABLE V.
Relations between Life Cycles and Chemical Composition.

Species.	Average length of gestation period.	Approximate length of life.	Conceptional age at maximum of third growth cycle.	Conceptional age at chemical maturity.	Total life at chemical maturity.	Composition at birth.		
						Water.	Protein.	Ash.
	days	yrs.	days	days	per cent	per cent	per cent	per cent
Cattle.....	285	25	850	435	4.6	76.3	18.4	4.56
Guinea pig.....	64	7	145	114	4.6	77.8	16.8	3.76
Man.....	285	80	5,300	1,285	4.4	82.0	14	3
Swine.....	120	20	200?	270-420	4.6	82	12-13	3
Dog.....	61	17		261	4.3	82	13-14	2
Cat.....	60	11		160?	3.9?	83	13-14	2.5
Rabbit.....	31	10	185			84	13	2-3
Rat.....	25	3-6	86	75	4.5	88	9.6	1.5
Mouse.....	20	3-6	62			86-87	11.0	1.7

SUMMARY.

The composition of animals should be compared on the fat-free basis in order to make apparent the effects of age or abnormal development.

On this basis it is shown that mammals show a rapid decrease in relative water content and increase in protein (nitrogen) and ash content from earliest life until the time of chemical maturity is reached. At this time the change becomes rather suddenly less, and nearly constant composition is shown.

Mammals vary in composition at birth. Those relatively mature have a low water content and those less mature, a high water content. The change in chemical development at birth follows the degree of physical development.

The fatness of the individual has no effect on the composition on the fat-free basis.

Edema, underdevelopment, and atrophy give abnormal water percentages.

Mammals reach chemical maturity at different ages, but these ages are a fairly constant relative part of the total life cycle.

A STUDY OF THE CONDITION OF SEVERAL INORGANIC CONSTITUENTS OF SERUM BY MEANS OF ULTRAFILTRATION.

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The inorganic constituents of the serum may be present in three forms: as ions, as undissociated molecules in equilibrium with the ions, and as non-ionizable compounds with some of the organic constituents. Owing to the number of ionic constituents present in the serum, only a few of the ionic species can be determined electrometrically (1). It has been proposed, however, to determine the sum total of any inorganic constituent present in the form of ions or undissociated molecules (which are potentially ions), *i.e.* the diffusible portion of the constituent, by means of compensation dialysis or ultrafiltration. The method of compensation dialysis which had been developed by Michaelis, Rona, and their students while satisfactory for the organic crystalloids of the serum gives variable results in the case of inorganic constituents because of the existence of a Donnan membrane equilibrium on the sides of the collodion membrane, as for instance in the chlorine determinations by this method of Rona and György (2).

The older determinations by means of ultrafiltration through Bechhold filters under pressures running into tens of atmospheres were open to criticism such as that of Burian (3) that any labile compounds between the proteins and inorganic ions may have been decomposed by the excessive pressures. Recently, however,

it has been found possible by a number of investigations to ultra-filter serum at pressures approximating systolic pressure (120 to 150 mm.). Cushny (4) used collodion sacs while Richter-Quittner (5) employed a patented Zsigmondy-Bachmann filter.

The work of Cushny is open to criticism because of its incompleteness and therefore lack of conclusiveness in several regards. Thus he determines the phosphate concentration in the ultra-filtrate, but not in the original serum and assumed Abderhalden's original determination of inorganic phosphorus in ox blood to be true for the sample with which he was working. No direct determinations were made on sodium and potassium; it was merely assumed that "the fact that the total ash of the filtrate is practically equal to that of the original serum in amount shows that the sodium and potassium pass through the membrane readily and in the same proportion as in the serum." Richter-Quittner obtained unusually high values for the calcium content of serum (40 to 46 mg. per 100 cc.) and extremely low values for the filterable calcium (2 to 3 mg. per 100 cc.), while Cushny found 60 per cent of the calcium to be diffusible and Neuhausen and Marshall (1) found 20 to 25 per cent of the total calcium to be in ionic condition, and this is only part of the diffusible calcium. Furthermore, Richter-Quittner has claimed that about 30 to 60 per cent of the potassium is non-filterable, while preliminary experiments done here had indicated that all the potassium is diffusible. In the case of constituents that are free in the serum, we should expect higher concentrations of the constituents in the filtrate than in an equal volume of the original serum, for a portion of the volume in the serum is occupied by the proteins instead of liquid containing these ions. Accordingly, it was deemed advisable to undertake a comparison of the contents of pig's serum and its ultrafiltrate as regards phosphate, chlorides, potassium, calcium, and sodium.

Apparatus and Method.

The ultrafilters were prepared according to the method described by Marshall and Vickers (6) except that when not in use the filters were kept in an ice chest and so we were enabled to use them repeatedly. The ultrafiltrates were tested and found free from protein.

30 cc. of serum were usually put into bags and filtered at pressures varying from 120 to 180 mm. of mercury, limits within which the pressure was found to have no effect on the character of the filtrate. 1 to 2 cc. were filtered in order to displace any water in the interstices of the bag with ultrafiltrate. This ultrafiltrate was discarded. A measured quantity of serum was then removed from the bag and ultrafiltration was resumed. After a quantity of ultrafiltrate sufficient for the analytical requirements had been obtained, ultrafiltration was stopped, a measured quantity of the residue in the bag was taken, and ultrafiltration continued into a second receptacle. Upon completion of the second ultrafiltration another sample was taken of the residue in the bag. Thus in most cases there were analyzed two ultrafiltrates, the original serum and the first and second residue. In a number of cases only one residue and one ultrafiltrate were analyzed.

For the determination of calcium the method of Kramer and Howland (7) was used; for potassium that of Kramer and Tisdall (8); for sodium that of the same authors (9); for phosphate, the Briggs modification of the colorimetric method of Bell and Doisy (10); and for chlorine, that of Whitehorn (11).

RESULTS AND DISCUSSION.

As no analyses for all constituents were made on the same sample of serum the results on the different constituents are represented separately. Several representative sets of results are given below expressed as mg. per 100 cc.

Phosphates.—In Table I are presented results on the inorganic phosphates.

From this table it may be seen that the ultrafiltrates have a higher concentration of inorganic phosphate than the sera from which they are filtered and also that the phosphate content decreases progressively in the residues. This state of affairs would be expected when the volume occupied by the proteins is taken into account and in the second column of each set there are shown the concentration that would be expected when the absence or increase in protein is taken into account. From Table I we may conclude that the inorganic phosphorus of the serum is present wholly in the form of ions or filterable electrolytes. This

result is of interest in connection with the work of Rona and Takahashi (12) who found by compensation dialysis that all the phosphate of horse serum is diffusible.

Chlorides.—In Table II are presented the results obtained on chlorides. It will be noted that as in the case of phosphate the chloride content per volume of the ultrafiltrates is greater than of the original serum or of the residues, and that the chloride

TABLE I.

Sample.	Set I.		Set II.		Set III.	
	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.
Original serum.....	11.8		11		10.9	
First filtrate.....	12.0	12.6	12	11.8	11.3	11.7
“ residue.....	11.1	11.6	10.7	10.7	10.8	10.7
Second filtrate.....	12.5	12.6	11.5	11.8	11.0	11.7
“ residue.....	11.0	11.4	10.6	10.5	9.5	10

TABLE II.

Sample.	Set I.		Set II.		Set III.	
	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.
Original serum.....	620		618		554	
First filtrate.....	687	674	693	675	594	600
“ residue.....	610	605	605	603	547	549
Second filtrate.....	683	674	680	675	603	600
“ residue.....	600	590			524	535

decreases as the protein content increases. The chloride concentrations found agree satisfactorily with those calculated when the changes in the volume occupied by the proteins are taken into account. The increase in chloride concentration of the filtrate found by us is in agreement with the work of Rusznyák (13) and in disagreement with Cushny and Richter-Quittner who obtained the same concentrations in the serum and its ultrafiltrate. These results likewise point to the conclusion that all the chlorine is present in a diffusible form in the serum, a conclusion which Rona (14) reached by means of compensation dialysis of horse serum and Asher and Rosenfeld (15) by diffusion of ox and rabbit sera against water, and Neuhausen and Marshall (1) through elec-

trometric determinations on dog serum. The existence of a fibrinogen-chlorine compound as postulated by Falta and Richter-Quittner (16) is very doubtful in view of the total diffusibility of the chlorine.

Potassium.—In Table III are presented the potassium analyses. It will be noted that within the limits of error of the analytical method the potassium is apparently all or nearly all diffusible when

TABLE III.

Sample.	Set I.		Set II.		Set III.	
	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
Serum.....	26		26		25	
First filtrate.....	26.4	28	25	28	25.2	27
Residue.....	26	25.3	24.7	25.3	28	24.5

we compare the calculated values with those actually found. In the case of human sera Richter-Quittner (5) had obtained results which indicated that from 40 to 75 per cent of the potassium was free. In the course of some preliminary results on ultrafiltration of human serum we obtained results that point to a complete diffusibility of potassium, the analysis giving 24, 22.4, and 22 mg. of potassium per 100 cc. in the original serum, ultrafiltrate, and residue, respectively.

TABLE IV.

Original solution.	First filtrate.	First residue.	Second filtrate.	Second residue.
400	395	400	400	400
40	40	40		
4.8	5	5		
9 in 0.1 N NaCl.	8.8	9.3	9.2	9.3

Before we commenced our work on the determination of *calcium*, we found it necessary to ascertain whether any lowering of the calcium ion concentration in the filtrate may not be due to adsorption of the calcium by the bag. We accordingly filtered aqueous solutions of CaCl_2 and CaCl_2 -NaCl mixtures through the bags. It was noted that there was no appreciable change in the calcium concentration of the filtrate as compared with the original material or the residues. Some of these results are presented in Table IV.

The results of the analyses for calcium are given in Table V.

As will be noted by comparing the concentrations of calcium found in the filtrates and residues with the values calculated on the assumption that all the calcium is diffusible, that only 40 to 62 per cent of the calcium is in a filterable condition. There is apparently a striking variation in the individual animals as to the quantity of filterable calcium. Unlike chlorine, phosphate, or potassium the residues are higher in calcium than in the original sera, as would be expected from the accumulation of some non-filterable compound. Our findings are in agreement with the conclusions of Rona and Takahashi (17) that about 75 per cent of the calcium in horse and pig sera is diffusible. Cushny (4) had found 60 to 70 per cent of the calcium in ox serum to be filterable. As mentioned in the introduction Richter-Quittner (5) had found

TABLE V.

Sample.	Set I.		Set II.		Set III.		Set IV.	
	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.
Original serum.....	11.8		10.8		10.4		11.2	
First filtrate.....	7	12.6	4.8	11.6	6.9	11.2	5.2	12.0
“ residue.....	13.2		11.7	10.6	12.8	9.6	11.0	10.6
Second filtrate.....	6.8	12.6						12
“ residue.....	14	11.4					13.2	9.7

only 5 to 7 per cent of the calcium to be filterable. His values for the total calcium in serum are evidently erroneous. As the result of several experiments we have found 40 to 50 per cent of the calcium of human serum to be filterable. It should also be noted that in the case of pig serum saturated at 40 mm. of CO₂ and also filtered in an atmosphere containing 40 mm. of CO₂ we failed to find any appreciable increase in the filterable calcium.¹

¹ These results are rather surprising in view of the well known equation of Rona and Takahashi (17) that

$$[\text{Ca}^+] = \frac{K_1[\text{H}^+]}{[\text{HCO}_3^-]} \quad (\text{I})$$

Since by the Henderson-Hasselbalch equation

$$[\text{H}^+] = \frac{K_2\text{pCO}_2}{[\text{HCO}_3^-]} \quad (\text{II})$$

That the *sodium* is totally diffusible has been accepted by all investigators who have worked on ultrafiltration. In this work the results obtained point likewise to a similar conclusion. Thus we obtained from a serum containing 336 mg. of sodium per 100 cc., 336 mg. in the filtrate and 317 mg. in the residue. While the filtrates did not always show an increase in the sodium content as might be expected, the residues were usually smaller in sodium content. Rona and György (2) have interpreted the variation in the titratable bicarbonate content of their ultrafiltrate with increase in the carbon dioxide tension as indicating that about 10 to 15 per cent of the sodium was present as a sodium-protein compound. However, direct analyses for sodium have indicated complete filtrability.

SUMMARY.

Analyses of pig sera, their ultrafiltrates and residues, indicate that the chlorides, phosphates, sodium, and potassium are totally diffusible. Potassium in human serum likewise seems to be totally diffusible. Calcium in pig serum seems to be from 30 to 50 per cent in a non-diffusible form.

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in which $p\text{CO}_2$ equals the partial pressure of CO_2 gas with which the solution is in equilibrium, the following relation is obtained:

$$[\text{Ca}^+] = \frac{K_1[\text{H}^+]^2}{K_2p\text{CO}_2} = \frac{K[\text{H}^+]^2}{p\text{CO}_2} \quad (\text{III})$$

Since the $[\text{H}^+]$ of serum is nearly proportional to the CO_2 tension for a given bicarbonate content, it follows that the ionic calcium is therefore filterable and should increase if there were any undissolved CaCO_3 in serum as implied in (I).

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A NEW PERMANENT STANDARD FOR ESTIMATION OF HEMOGLOBIN BY THE ACID HEMATIN METHOD.

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(Received for publication, June 4, 1923.)

While investigating various hemoglobin methods, we found the acid hematin modification¹ of Palmer's method² very satisfactory in as far as accuracy of estimation is concerned. The preparation of an accurate stock solution of the strong acid hematin standard is a difficult task for those who are not research biochemists. Since the solution must be prepared about every 4 months, the method is hardly suitable for general use by physicians. We realized that a permanent standard is required to make the method universally available.

Newcomer's method,³ using a colored plate of glass as the standard, seemed very attractive. But we found, as did Miss Robscheit,¹ that the color is too pale to give consistent results at least with workers whose eyes are not sensitive to very slight changes of pale colors.

We have, therefore, devised a standard solution containing inorganic material only, which if set at 15 mm. in a colorimeter will match the color of a 1 per cent acid hematin solution set at 10 mm. (a 1 per cent solution being a 1 in 100 dilution in weak HCl of blood containing 13.8 gm. of hemoglobin in 100 cc.). The intensity of the color varies with change in temperature, but this is taken into account in the calculation (see below). The final standard prepared by us contains 32 gm. of ferric sulfate and 80 mg. of chromic sulfate in 100 cc. Unfortunately, the ferric

¹ Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489. Robscheit, F. S., *J. Biol. Chem.*, 1920, xli, 209.

² Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

³ Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 489.

sulfate on the market is not uniform in color, we found a great difference in the character of the color of several samples secured from the same manufacturer. We made our ferrie sulfate from recrystallized ferrous sulfate by the usual method, but we carefully regulated the amount of heat used.⁴ We have every reason for expecting our standard solution to keep indefinitely. We have been unable to detect any change in color during 10 months.

TABLE I.

Comparison of Estimations of Hemoglobin by the Proposed Method and by Van Slyke's Method.

Blood No.	Percentage of hemoglobin.	
	New method.	Van Slyke's method.*
1	29.7	30.6
2	37.0	36.8
3	43.7	42.9
4	49.7	49.0
5	56.0	56.0
6	61.0	61.3
7	65.7	66.0
8	72.3	74.0
9	80.4	79.8
10	90.8	91.9
11	96.2	96.7
12	100.5	100.0
13	104.8	104.6
14	117.3	116.8
15	123.2	122.6
16	131.3	132.2

* We found it necessary to take the mean of several Van Slyke estimations in order to keep the probable error below 1 per cent. The results by our method given above are also the mean of several determinations.

Estimations made by using this standard agree very closely with the results by Van Slyke's method⁵ as will be seen by examination of Table I. No other method that is adapted to clinical use gave results within 5 per cent of the Van Slyke estimations.

⁴ Because of this difficulty in reproducing our standard we shall check the solution before it is distributed. It may be secured from Hynson, Westcott and Dunning, Baltimore.

⁵ Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

Technique.—If the patient bleeds freely from a skin puncture, 0.05 cc. of blood may be taken with an accurate 0.1 cc. pipette. After wiping off the tip of the pipette the blood is quickly blown out into 2.45 cc. of water in a test-tube and mixed at once. The tube may be set aside (corked) until a convenient time for estimation, or the work may proceed as soon as the blood is fully laked.

TABLE II.

Per Cent of Hemoglobin Corrected for the Temperature of the Standard, Corresponding to Various Colorimeter Readings.

Colori- meter reading.	Temperature.										
	15.5°	16.5°	17.5°	18.5°	19.5°	20.5°	21.5°	22.5°	23.5°	24.5°	25.5°
<i>mm.</i>											
8.0	131.5	134.1	136.7	139.5	142.0	144.7	147.4	150.8	154.0	157.0	160.6
8.5	123.7	126.2	128.6	131.1	133.6	136.2	139.2	142.0	144.8	148.0	151.2
9.0	116.9	119.2	121.3	123.8	126.2	128.8	131.4	133.9	136.5	139.1	142.7
9.2	114.5	116.7	119.0	121.2	123.5	125.9	128.3	131.3	133.9	136.9	139.8
9.4	111.9	114.1	116.3	118.5	120.8	123.3	125.8	129.4	131.2	133.9	136.6
9.6	109.4	111.6	113.9	116.1	118.4	120.6	122.8	125.4	128.5	131.1	133.8
9.8	107.2	109.3	111.4	113.6	115.8	118.1	120.5	123.3	126.2	128.6	131.0
10.0	105.0	107.1	109.2	111.5	113.5	115.8	118.2	120.5	123.1	125.6	128.2
10.2	103.0	105.0	107.0	109.1	111.2	113.5	115.9	118.1	120.5	123.1	125.7
10.4	101.0	103.0	105.0	107.0	109.0	111.1	113.2	115.6	118.0	120.6	123.2
10.6	99.0	101.1	103.0	105.0	107.0	109.0	111.0	113.3	116.0	118.4	120.8
10.8	97.4	99.4	101.4	103.3	105.2	107.2	109.3	111.6	113.9	116.2	118.7
11.0	95.8	97.6	99.4	101.2	103.0	105.0	107.0	109.2	111.5	114.0	116.5
11.5	91.3	93.2	95.1	96.9	98.8	100.5	102.5	104.7	106.9	109.1	111.5
12.0	87.8	89.5	91.2	92.9	94.7	96.5	98.4	100.4	102.5	104.6	106.8
12.5	84.2	85.9	87.6	89.2	91.2	92.8	94.5	96.4	98.5	100.5	102.5
13.0	80.8	82.5	84.3	85.9	87.5	89.2	91.0	92.8	94.7	96.8	98.9
13.5	78.0	79.5	81.0	82.6	84.2	85.8	87.5	89.4	91.3	93.2	95.1
14.0	75.2	76.7	78.2	79.7	81.3	82.8	84.3	86.1	88.0	89.9	91.8
15.0	70.2	71.6	73.0	74.4	75.8	77.3	78.6	80.4	82.2	83.9	85.4
16.0	65.8	67.1	68.4	69.7	71.0	72.4	73.8	75.4	77.0	78.6	80.3

Add exactly 2.5 cc. of 0.2 N HCl (18 cc. of c.p. acid per liter are close enough). The volume of the mixture is 5 cc., and the dilution of the blood is 1 part in 100. Warm the tube in a water bath at 55–60° (often hot tap water is satisfactory) for 7 minutes or more. This develops the maximum color of the acid hematin (we did not find 1 hour standing at room temperature sufficiently reliable). In the meantime the colorimeter has been set and tested

for uniformity of the light on both sides. Put the standard solution in the left hand cup and set at 15 mm. Cool the blood mixture and put it in the other cup. Make several readings, being careful to avoid eye-strain. Determine the temperature of the standard at once by inserting a thermometer in the liquid.

No calculation is necessary since the percentage of hemoglobin can be read directly from the table using the average reading and the temperature. Table II is an abbreviation of the complete table that will be furnished with the solution. An estimation of 100 per cent indicates that the blood contains 13.8 gm. of hemoglobin in 100 cc. Normal bloods will, of course, give a distinctly higher estimation.

It is more satisfactory to use oxalated venous blood. In this case measure exactly 1 cc. into a 100 cc. flask, containing about 40 cc. of water. After laking has occurred add 50 cc. of 0.2 N HCl while mixing, and dilute to the mark. To cut the foam use a drop of alcohol (caprylic alcohol must not be used). Mix well, and heat some of the solution in a test-tube as described above.

In cases of marked anemia use double quantity of blood, and divide the estimated per cent of hemoglobin by 2. If 0.1 cc. is taken for the micro method 2.45 cc. of HCl are to be used instead of 2.5 cc.

Preservation of the Standard.—The solution is kept in a Non-sol or Pyrex flask, corked with a rubber stopper (if a new stopper is used, scour off the powdered material). The standard after being used is stored in a second flask. Before the main stock of standard is exhausted check up the used standard against it by making estimations with both, using the same acid hematin preparation. If they agree, pour the used standard into the other flask and use again. We have found that with reasonable care no change in the solution is noticed during months of repeated use. Of course, the cup and plunger of the colorimeter must be clean and dry before the standard is put in the cup. If dried salts collect on the stopper and on the mouth of the flask, wipe off with a damp cloth.

Advantages of the Method.—The technique is quick, simple, and accurate. Special apparatus is not required. The standard is permanent.

A NEW PERMANENT STANDARD FOR SAHLI'S HEMOGLOBINOMETER.

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(Received for publication, June 4, 1923.)

Under optimum conditions the estimations of hemoglobin by Sahli's method are surprisingly accurate. If the diluting test-tube is accurately graduated and has the same inside diameter as the standard tube, and if the blood pipette has the proper capacity, very satisfactory determinations can be made provided the standard solution is correct.

The manufacturers can readily fulfil all the requirements except the last one. The unreliability of the standard has brought the method into disrepute. Acid hematin standards deteriorate and soon become worthless. I have examined permanent standards made by two leading manufacturers. Those of one firm have a color which does not resemble that of acid hematin. Estimations made with these standards showed that they did not have the hemoglobin equivalence that was claimed for them. The standards of the other firm were better, but they became darker after some months. They were discontinued by the manufacturer.

The writer has been able to prepare a permanent standard from inorganic material that has shown no change in 10 months. It is made by mixing 50 cc. of ferric sulfate solution (containing 53.3 gm. in 100 cc.), 15 cc. of cobalt sulfate solution (10 gm. $\text{Co SO}_4 \cdot 7\text{H}_2\text{O}$ in 100 cc.), and 10 cc. of water. Unfortunately, ferric sulfate (either in dry form or as a solution) of uniform color value seems to be unobtainable. Different samples secured from the same manufacturer varied widely in the character of the color. If one wishes to duplicate my standard, he must check it up against 1 per cent acid hematin that has been prepared with research accuracy. It is probable that different proportions of ferric sul-

fate and cobalt sulfate than those given above will have to be used.¹

Technique.—Put (approximately) 0.2 N HCl in the graduated test-tube to about the 10 mark. Deliver 0.02 cc. of blood with the pipette into the solution and mix quickly. Draw the liquid into the pipette twice blowing it out, then rinse the tip with a drop of the acid solution. Keep the test-tube in a beaker of hot water (55–60°) for 7 minutes. In that length of time the full acid hematin color is developed—1 hour at room temperature did not prove to be as reliable as some investigators claim.

TABLE I.

Comparison of Hemoglobin Estimations by Van Slyke's Method and by Sahli's Method Using the New Permanent Standard.

Blood No.	Hemoglobin.	
	Van Slyke's method.	New method.
	<i>per cent</i>	<i>per cent</i>
1	96.7	97.0
2	99.7	99.0
3	104.6	104.0
4	114.5	115.0
5	117.1	117.0
6	119.3	120.0
7	122.6	123.0
8	122.0	123.0
9	132.0	132.0

Cool the tube and dilute the mixture gradually with careful mixing until it matches the standard. The standard must be at 19–20° to give strictly accurate results. Immerse the standard tube in water at that temperature for a few minutes before using it, if the room temperature is different.

At higher temperatures the standard becomes darker (increased hydrolytic dissociation) and underestimation results. The error is approximately 1 per cent for each degree.

¹ Because of the difficulty of reproducing the standard, we shall check up the solution before it is distributed. Hynson, Westcott and Dunning of Baltimore, will furnish the material.

The method was carefully standardized against Van Slyke's method.² A reading of 100 on the graduated tube indicates that the blood contains 13.8 gm. of hemoglobin in 100 cc. The readings will not, therefore, be in terms of percentage of the average normal. The work of Meyer and Butterfield³ seems to show that the Sahli readings will average 116 for normal males and 109 for females provided the blood contains 5 million red cells per cubic millimeter.

When the method is carried out with great care the estimations are almost as accurate as those by Van Slyke's method, as will be seen by examining Table I. Routine estimations should give results within 2 per cent of the correct amount.

SUMMARY.

A permanent standard made of inorganic materials is proposed for the Sahli hemoglobinometer. The estimations are accurate if the pipette and tubes are correctly calibrated.

² Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

³ Meyer, E., and Butterfield, E. E., *Arch. Int. Med.*, 1914, xiv, 94.

DIET AND THE BORDER-LINE OF ACETONURIA.

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(Received for publication, May 28, 1923.)

In a previous paper (Hubbard and Wright, 1922), the acetone excretion of subjects who were receiving diets low in carbohydrate and high in fat was studied. Each subject received a number of different diets in which the proportion of these foods varied, and it was found that when more than 1 molecule of ketogenic material was burned in the body for each molecule of antiketogenic material burned simultaneously there was an increased excretion of the acetone bodies, and these were occasionally slightly increased when relatively less ketogenic material was burned (Shaffer, 1921). Four experiments on arthritic patients are described in which similar diets were fed, and the diets were kept constant throughout the periods.

Protocols.

Case 1, Hospital No. 7500.—Female, 42 years old, 66 inches tall, weight 160 pounds. Her basal metabolism was 1,465 calories per day. She was fed a diet consisting of 45 gm. of protein, 65 gm. of carbohydrate, and 145 gm. of fat. This diet furnished 1,758 calories, or 20 per cent more than her basal requirement. 10 per cent of the calories were fed as protein, 14.2 per cent as carbohydrate, and the balance as fat. This diet contains 1.1 molecules of ketogenic for each molecule of antiketogenic material.

Case 2, Hospital No. 8034.—Female, 49 years old, 61.5 inches tall, weight 112.5 pounds. Her basal metabolism was 1,224 calories. She was fed 37 gm. of protein, 55 gm. of carbohydrate, and 122 gm. of fat. This diet furnished 1,466 calories, or 20 per cent more than her basal requirement. 10 per cent of the calories were fed as protein and 15 per cent as carbohydrate. The diet contained 1 molecule of ketogenic for each molecule of antiketogenic compounds.

Case 3, Hospital No. 7726.—Female, 55 years old, 65 inches tall, weight 136.5 pounds. Her basal metabolism was 1,528 calories per day. She was fed 45 gm. of protein, 75 gm. of carbohydrate, and 180 gm. of fat. This diet

contained 2,100 calories, or 37 per cent more than her basal requirement. 8.6 per cent of the calories were fed as protein, and 14.3 per cent as carbohydrate. This diet contained 1.1 molecules of ketogenic for each molecule antiketogenic material.

Case 4, Hospital No. 8089.—Male, 40 years old, 66.5 inches tall, weight 114.5 pounds. His basal metabolism was 1,438 calories per day. He received 43 gm. of protein, 43 gm. of carbohydrate, and 153 gm. of fat. This diet furnished 1,621 calories, or 20 per cent more than his basal requirement. 10 per cent of the calories were furnished as protein, and the same amount as carbohydrate. The diet contained 1.3 molecules of ketogenic for each molecule of antiketogenic material.

The basal metabolism of each patient was determined by the Benedict portable respiration calorimeter (Benedict, 1918) and the probable metabolism of the patient roughly estimated from the value so obtained and his activity. As the physical condition of all the patients limited the activity, the metabolism in all cases was lower than it would have been for normal subjects of the same height and weight. Food was furnished to supply the calories so estimated. The ratios between ketogenic and antiketogenic material in the different diets were calculated from a slightly modified form of Woodyatt's (1921) formula. Acetone bodies were determined by a method recently published (Hubbard, 1921). As some of the subjects had been receiving diets high in fat as part of their treatment before the periods were commenced, satisfactory control determinations could not be obtained in all cases. A series of determinations upon one of the subjects on a normal diet is given to show the variations found from day to day (Table I).

A slight acetonuria developed in all cases as shown by quantitative determinations and confirmed in most instances by the sodium nitroprusside test, but the amounts found in the urine when the diets contained 1 molecule of ketogenic for each molecule of antiketogenic material were very small. The average amounts excreted by the different subjects varied slightly, but these variations did not correspond with the differences in the amount of fat fed, or, as far as could be determined, with the estimated activity of the patients. The subject who received a diet which contained 1.3 molecules of ketogenic for each molecule of antiketogenic material excreted on the average ten to twenty times as much acetone as did the others, but the actual amounts contained in the urine were small. There was no significant change in the rate

TABLE I.

Urine.

Series No.	Date.	Volume.	Acetone bodies.			Remarks.
			Acetone + diacetic.	β -Hydroxybutyric acid.	Total.	
	1921	cc.	mg. per 100 cc.	mg. per 100 cc.	gm.	
1	Dec. 8	1,140	0.7	1.7	0.027	Patient on a diet high in fat before the experimental period. Experimental diet contained 1.1 molecules ketogenic for each molecule antiketogenic. Diet furnished 20 per cent more calories than basal need. Diet stopped after Dec. 19.
	" 9	1,300	1.7	2.0	0.048	
	" 10	1,700	0.2	1.2	0.023	
	" 11	680	1.5	3.0	0.032	
	" 12	580	1.3	2.1	0.020	
	" 13	840	3.2	2.8	0.051	
	" 14	480	2.8	3.5	0.030	
	" 15	735	6.7	7.1	0.103	
	" 16	780	6.3	3.1	0.083	
	" 17	570	6.0	5.2	0.066	
	" 18	760	4.4	5.3	0.072	
	" 19	735	3.4	4.1	0.080	
	" 20	1,493	5.6	7.35	0.192	
2	" 21	560	1.8	3.3	0.028	Patient placed on a diet Feb. 24. Diet contained 1 molecule ketogenic for each molecule antiketogenic material. Diet furnished 20 per cent more than her basal requirement.
	" 22	930	2.0	0.4	0.022	
	" 23	1,000	0.3	1.1	0.014	
	1922					
	Feb. 22	1,880	0.6	0.9	0.028	
	" 23	2,100	0.6	0.6	0.024	
	" 24	1,600	0.2	0.3	0.008	
	" 25	1,880	1.1	1.2	0.056	
	" 26	1,790	1.6	1.3	0.053	
	" 28	1,940	1.7	1.2	0.057	
	Mar. 1	1,380	3.9	1.2	0.070	
	" 3	1,880	2.1	1.7	0.070	
	" 4	1,900	1.3	1.1	0.045	
	" 6	1,900	1.8	0.8	0.048	
3	" 10	1,840	0.6	0.5	0.020	Patient on a diet high in fat before the experimental period. Experimental diet
	" 11	2,885	0.75	0.2	0.027	
	" 12	1,900	1.75	1.0	0.063	
	" 13	1,440	2.4	1.3	0.053	
	1921					
	Dec. 8	900	0.7	1.9	0.023	
	" 9	1,850	0.9	1.2	0.038	
3	" 10	1,390	1.9	2.2	0.058	

Results of the determinations of the acetone bodies are expressed in terms of acetone.

TABLE I—*Concluded.*

Series No.	Date.	Volume.	Acetone bodies.			Remarks.
			Acetone + diacetic.	β -Hydroxybutyric acid.	Total.	
	1921	cc.	mo. per 100 cc.	mo. per 100 cc.	gm.	
	Dec. 11	2,000	2.2	2.2	0.087	contained 1.1 molecules ketogenic for each molecule antiketogenic material. Diet furnished 37 per cent more calories than her basal requirement.
	" 12	1,100	3.0	3.4	0.072	
	" 13	1,410	3.3	3.1	0.089	
	" 14	1,200	3.4	3.7	0.090	
	" 15	1,395	3.1	4.4	0.105	
	" 16	1,563	2.3	2.45	0.074	
	" 17	1,240	3.1	4.1	0.089	
	" 18	985	3.1	3.2	0.063	
	" 19	1,480	2.6	1.6	0.063	
	" 20	1,500	0.7	1.7	0.035	
	" 21	1,610	2.1	1.5	0.058	
	" 22	1,280	2.6	1.8	0.056	
	" 23	1,240	2.3	2.0	0.053	
	1922					
4	Feb. 20	820	1.2	0.8	0.016	A number of determinations were made on this patient before the experimental period was begun. Diet started Mar. 10. Diet contained 1.3 molecules ketogenic for each molecule antiketogenic material. Diet furnished 20 per cent more than his basal requirement.
	" 23	620	1.3	2.7	0.025	
	" 24	600	0.6	2.3	0.016	
	" 25	700	0.8	1.1	0.012	
	" 26	1,480	0.4	1.0	0.016	
	" 27	1,330	0.3	0.0	0.004	
	" 28	2,105	0.4	0.3	0.014	
	Mar. 2	1,600	0.8	2.1	0.046	
	" 3	1,860	0.2	0.9	0.020	
	" 9	780	0.4	0.8	0.009	
	" 10	865	0.7	1.6	0.019	
	" 12	540	31.6	20.2	0.180	
	" 13	580	39.6	25.1	0.394	
	" 14	850	16.2	14.7	0.262	
	" 15	1,168	13.7	4.5	0.212	
	" 16	1,360	16.2	14.3	0.414	

at which acetone was excreted during the periods of study. These results show that the border-line of ketosis is found in normal subjects when the diet contains 1 molecule of antiketogenic for each molecule of ketogenic material. Patients receiving such diets should be carefully observed, and the development of excessive acetonuria watched for (Mason, Richardson, and Ladd, 1923).

Shaffer (1922) has recently stated that a ratio of 2 molecules of ketogenic for each molecule of antiketogenic material probably represents the conditions under which the acetone bodies are produced, and has shown that if this ratio is assumed when diets very low in carbohydrate are fed the degree of acetone excretion can be predicted with great accuracy. In none of the experiments presented here should acetone have been excreted if this relationship is assumed. On the other hand, in the fourth experiment 8.6 gm. of acetone, or approximately twenty times as much acetone as was found, should have been excreted if 1 molecule of ketogenic to 1 of antiketogenic material represents conditions under which the acetone bodies are produced. Even greater discrepancies are shown by some of the experiments of Hubbard (1923) and Hubbard and Wright (1922).

In the opinion of the writers Shaffer's calculations prove that when very little antiketogenic material is included in the foods metabolized 1 molecule of it will burn 2 molecules of ketogenic material, because agreement would not have been found between the actual and theoretical excretion of acetone if the factors which he assumed in making the calculations had not been at least approximately correct, or if much more glycogen had entered into metabolism than was taken into account in making the calculations (Mason, Richardson, and Ladd, 1923; McCann and Hannon, 1923). It is possible that the body can burn 2 molecules of ketogenic with 1 molecule of antiketogenic material, but that it does not do so except when the mixtures of foods metabolized are very low in antiketogenic compounds (as in all the experiments discussed by Shaffer); and that ordinarily it utilizes relatively more antiketogenic material for this purpose. Such an assumption would explain both the findings of Shaffer and the results of such experiments as are described above, but it does not seem necessary to make it. Shaffer (1922) and Hubbard and Wright (1922) have suggested that when the metabolism of the organism as a whole is below but near the border-line of ketosis, a formation of excessive amounts of acetone occurs through variations in the mixtures of foodstuffs metabolized at different times of the day and in different parts of the organism, and it appears to the writers that such an explanation is adequate to explain such acetonuria as is found when foods metabolized contain less antiketogenic material than

is indicated as the border-line of ketosis by the ratio proposed by Shaffer.¹

In calculating the amounts of glucose derivable from protein in excess of that needed to burn the acetoacetic acid derived from the same protein Hubbard and Wright assumed that 1 molecule of glucose was equivalent to 1 molecule of acetoacetic acid, and the result so calculated formed part of the formula used by them in studying ketosis. Since there appears to be doubt whether such an assumption is valid, more general mathematical expressions similar to those suggested by Woodyatt (1921) and by Wilder and Winter (1922) should be used in studying the problem of ketogenesis.

SUMMARY.

Diets containing between 1 and 2 molecules of ketogenic for each molecule of antiketogenic material were fed to four arthritic subjects, and the amount of the acetone bodies excreted in the urine was determined. While there was an excretion of the acetone bodies the amounts found were small, and could be explained by Shaffer's theories of ketogenesis.

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¹ In two cases (A376588 and A375561) suffering from afebrile infections Wilder and Winter (1922) found an excretion of fairly large amounts of acetone when the foods burned contained, by their calculation, less than 1 molecule of antiketogenic for each molecule of ketogenic material, but the acetone excretion of these patients was much less than that calculated from the 1:1 ratio. It seems to the writers that in these cases there was an exaggeration of the irregularities in metabolism discussed, which accounts for the findings. In other cases when the foods burned have given ratios between the 2:1 and the 1:1 ratio, excretion of only small amounts of acetone has been reported.

STUDIES IN NUCLEIN METABOLISM.

I. ADENINE NUCLEOTIDE IN HUMAN BLOOD.

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In 1914 Bass (1) showed that other purines than uric acid circulate in normal human blood and suggested that nucleotides might be present. He also obtained a picrate which on the basis of its melting point he took to be adenine picrate. Greenwald and Gross (2) have recently confirmed the finding of the picrate which, in the opinion of these latter authors, is a "mixture of hypoxanthine and adenine picrates."

In 1920 Thannhauser and Czoniczner (3) claimed to have discovered nucleotides in human blood, but the method they give for the determination of these substances is one which could hardly be applied clinically, as it requires about 100 cc. of blood and involves a double protein precipitation. Furthermore, the evidence they give for the presence of nucleotides is not entirely convincing. They precipitated 300 cc. of serum (from what source they do not state) with heat and sulfosalicylic acid and evaporated the filtrate to a small volume and treated it with copper sulfate and sodium bisulfite. The copper precipitate so obtained was decomposed with hydrogen sulfide and evaporated in vacuum to a small volume. This material gave a strong "yellow-red" murexide test. Another portion was taken up in hot 5 per cent mineral acid and was found to give a dark red color with resorcinol and a brown-red color with orcinol. Ammonium molybdate gave a "strongly positive" reaction for phosphate. They conclude from these reactions and from their quantitative method that the copper precipitate contains nucleotide to the extent of about 15 mg. per 100 cc. of serum. It may with some reason, however, be objected that the positive murexide test might be due to the uric acid,

which, of course, is known to be present, and would be precipitated by the copper. The red color with resorcinol indicates a ketose sugar and the naturally occurring pentoses are aldo pentoses. Again, it is possible that the phosphate indicated by the ammonium molybdate test might have been carried down with the copper as simple phosphate as there is no direct evidence presented that it was bound and the test is a very delicate one. In any event, further evidence on this interesting point is perhaps worth while.

In 1922 Davis, Newton, and Benedict (4) proved beyond any question the presence of considerable amounts of uric acid bound to *d*-ribose and showed that this compound occurs in small quantities in human blood.

Interest in combined purines in the blood was thus aroused and it seemed more than likely that normal human blood would contain certain of the higher decomposition products of nucleic acid as well as the final cleavage products such as uric acid, around which the attention of biological chemists had previously been centered. And it is important for a complete understanding of the purine metabolism that these intermediate products should be discovered, if indeed they are present, and that a simple method for their determination should be found.

With a view to proving the presence or absence of nucleotides in the blood the author undertook a study of the subject during the winter of 1921-1922 at the Presbyterian Hospital, New York, under the Department of Medicine of the College of Physicians and Surgeons, Columbia University. The work was unavoidably interrupted and was resumed again in November, 1922, at the Thorndike Memorial Laboratory of the Boston City Hospital.¹ While the work is by no means considered finished it seems advisable, in view of the interest in the intermediate products of nuclein metabolism, to publish the work so far done.

Certain qualitative tests first indicated the presence of a nucleotide in human blood. Tungstic acid blood filtrate was treated with an excess of silver lactate in lactic acid, the resulting precipitate was broken up and the filtrate treated with neutral lead acetate in neutral or slightly acid solution. The resulting lead precip-

¹ The author wishes to thank Dr. Otto Folin for the privilege of working in his laboratories during the construction of the Thorndike Memorial Laboratory.

itate was decomposed with hydrogen sulfide and the filtrate from lead sulfide gave a positive pentose reaction with orcinol (violet color). With naphthoresorcinol the blueish color did not shake out with ether or benzene. The substance giving the tests was, therefore, a pentose and not glycuronic acid.

If, previous to the silver precipitation, the blood filtrate was hydrolyzed in a steam bath with 2 per cent sulfuric acid and the same procedure as outlined above was then carried out, no trace of pentose reaction could be found. Again, if the blood filtrate was precipitated with silver and then with lead and the filtrate from lead sulfide was hydrolyzed with 2 per cent sulfuric acid for 2 hours in a steam bath, subsequent addition of silver, after proper neutralization, precipitated the nitrogen containing part of the substance, while the material producing the pentose reaction remained in the filtrate.

Reactions such as these would be expected if a nucleotide were present. It would be precipitated by silver (in part at least) and be evidenced by a sugar reaction, but if the blood filtrate were first subjected to acid hydrolysis the purine-sugar-phosphoric acid complex would be broken down and the sugar, of course, would not be precipitated with silver. Preliminary evidence of a qualitative sort was thus obtained that blood contains nucleotides.

In order to prove the presence of a nucleotide one must either isolate the substance as such and subject it to proper analysis or one must isolate some of the decomposition products of the nucleotide from a precipitate which could contain those decomposition products *only* if they were combined together in the form of a nucleotide. It is along these latter lines that the author has been working.

If to the protein-free tungstic acid filtrate of Folin and Wu (5) one adds a 20 per cent solution of uranyl nitrate, a flocculent precipitate results which when properly decomposed is found to contain nitrogen. This precipitate is believed to contain bound adenine, probably in the form of nucleotide. Adenine nucleotide is quantitatively precipitated by uranium nitrate in slightly acid solution provided there is a small amount of simple phosphate present also. Neither nucleosides nor free bases are so precipitated in dilute solution. If adenine nucleotide is present in blood it should be found in the uranium precipitate, comparatively free from other substances.

For a long time it was attempted to isolate adenine nucleotide or some of its decomposition products from the uranium precipitate obtained by treating the tungstic acid filtrate with uranyl nitrate. Attempts were only partially successful, however, as the yields were very small, and it was finally seen that the small amounts of tungstic acid in the blood filtrate seriously interfered with the subsequent procedures and prevented the quantitative separation of the component parts of the molecule. Picric acid was then used as a protein precipitant and was found to serve admirably.

500 cc. of whole blood from a chronic nephritic were diluted with 2,000 cc. of water, 40 gm. of solid picric acid were added, and the mixture was shaken until protein precipitation was complete. The clear yellow filtrate was treated with 125 cc. of 20 per cent uranyl nitrate, and after $\frac{1}{2}$ hour the flocculent precipitate was centrifuged off and washed four times with 80 per cent ethyl alcohol, saturated with sodium chloride. The precipitate was then dissolved in 50 cc. of 5 per cent (volume) sulfuric acid. A small portion representing 50 cc. of blood was rapidly neutralized and made alkaline with concentrated ammonia and centrifuged until clear. 10 per cent silver nitrate in ammonia produced absolutely no precipitate in this solution. There were, therefore, no free purines present.

The remainder of the solution (representing about 450 cc. of blood) was heated in a steam bath for 2 hours under a reflux condenser. The solution was then made alkaline with concentrated ammonia and centrifuged until clear. In this clear liquid 10 per cent silver nitrate in ammonia produced a voluminous gelatinous precipitate characteristic of silver purine. This was centrifuged off, washed with water, decomposed with hydrogen sulfide, and the filtrate from the silver sulfide freed from hydrogen sulfide by boiling and made acid to methyl red with HCl. While still hot, saturated picric acid was run in. As the solution cooled a mass of fine, feathery needles separated out. These were centrifuged off, washed with cold water, and recrystallized from hot water. The melting point of the pure product was 279° C. It was almost insoluble in cold water, easily soluble in hot water and in alcohol: On analysis it proved to contain 30.00 per cent nitrogen. Theoretical for adenine picrate (without water of crystallization) 30.79 per cent nitrogen. The yield of crude material was about 95 mg.

The filtrate from the original picric acid precipitation (after removal of the adenine picrate) produced with silver nitrate only the slightest precipitate—about the amount one would expect from a solution saturated with adenine picrate. Adenine formed, therefore, by far the largest part of the bound purine present in the silver precipitate, if not its entirety.

These data seem to prove that the gelatinous silver precipitate was adenine silver and its appearance after hydrolysis in sharp

contrast to its absence before hydrolysis proves that the adenine was bound.

The filtrate from the first silver precipitate was treated with HCl to remove the silver and was then found to give strong orcinol and phloroglucin reaction, indicating the presence of pentose.

The uranium precipitate from another sample of blood was treated with 30 cc. of 5 per cent ammonium acetate and then with 250 cc. of 10 per cent ammonia and the resulting mixture was boiled hard and filtered through double filters. The filtrate was evaporated to 30 cc. and enough ammonia was added to make the solution 3 per cent. It was then autoclaved under pressure at 150° C. for 2 hours. If nucleotides were present they should be changed to nucleosides. With this in mind the cooled solution was made barely acid with acetic acid and an excess of neutral lead acetate added to remove the simple phosphates and any unchanged nucleotide. A voluminous precipitate resulted and was filtered off. This precipitate was insoluble in acetic acid and was presumably lead phosphate. To the filtrate was added, drop by drop, dilute ammonia. Nucleosides are precipitated by lead acetate and ammonia. A dense flocculent precipitate resulted immediately. This was washed and decomposed with hydrogen sulfide. To the filtrate from the lead sulfide was added enough sulfuric acid to make a 5 per cent solution and the mixture heated on a steam bath for 2 hours. It was then cooled, made alkaline with ammonia, and treated with silver nitrate in ammonia. A dense gelatinous precipitate was formed which was washed with water and decomposed with hydrogen sulfide. The filtrate was treated as outlined above with picric acid. A crystalline precipitate was formed which proved to be adenine picrate identical with that produced in the first experiment.

Adenine picrate had thus been recovered first by acid hydrolysis of the uranium precipitate and second by acid hydrolysis of the alkaline lead salt obtained after alkali hydrolysis of an ammoniacal extract of the same uranium salt.

A strong pentose reaction was found in the filtrate from the silver precipitate just as in the first experiment.

This train of reactions is one that would be given by adenine nucleotide. Adenine nucleotide is liberated somewhat imperfectly by ammonia from its uranium salt. On alkali hydrolysis under pressure at 150°C. it would be transformed into adenine nucleoside. The addition of lead acetate in slightly acid solution would remove all the simple phosphates and any undecomposed nucleotide, while the nucleoside would pass into the filtrate and be thrown down by lead in alkaline solution.

It can readily be shown that under the conditions of the experiment neither adenine nor adenine nucleoside are precipitated by uranium nitrate, while adenine nucleotide is quantitatively removed. Therefore, the finding of bound adenine and a pentose reaction in the uranium precipitate indicates strongly that the adenine was present originally as nucleotide. This position is further strengthened when the alkaline extract of the precipitate yields after hydrolysis under pressure a substance precipitating with alkaline lead only and when adenine is recovered from this second substance (presumably the corresponding nucleoside) on acid hydrolysis. Indeed this conclusion seems fairly clear unless we postulate the existence of adenine bound in some form other than nucleoside or nucleotide.

If this be the case—if indeed the uranium precipitate does contain adenine nucleotide—it should be possible to demonstrate not only bound adenine but also bound phosphate. With this end in view the following experiment was undertaken.

50 cc. of normal blood were precipitated with picric acid as described above. Two 50 cc. portions of the filtrate (representing 10 cc. of blood each) were treated separately with uranium nitrate and the precipitates centrifuged down in two 15 cc. tubes. To one precipitate were added 10 cc. of 7 per cent (volume) sulfuric acid and the mixture was stirred until clear. The tube with its contents was then heated in a steam bath for 2 hours. A small funnel in the mouth of the tube prevented any serious loss of water. The solution was then cooled to room temperature. 10 cc. of 7 per cent cold sulfuric acid were then added to the *other* precipitate and stirred until solution was complete. The two acid solutions (hydrolyzed and unhydrolyzed) were then transferred quantitatively to two 50 cc. volumetric flasks and to each were added in succession 10 cc. of molybdate solution,² 1 cc. of 0.5 per cent hydroquinone, and 1.0 cc. of 20 per cent sodium sulfite. The flasks were allowed to stand for $\frac{1}{2}$ hour. The method here used is essentially the blood phosphate method of Bell and Doisy as modified by Briggs (6) with such changes as are necessary for this particular experiment. At the end of half an hour the unhydrolyzed sample showed a rather pale blue-green color, while the hydrolyzed sample was deep blue with a greenish tinge. The increase in color developed in the hydrolyzed sample corresponded to about 0.2 mg. of phosphorus. It would be impossible to estimate by this method the exact amount of free phosphate in the

² 4.8 gm. of ammonium molybdate are dissolved in 60 cc. of water. To this solution are added 40 cc. of water containing 7.6 cc. of concentrated sulfuric acid.

two samples owing to the variable amount of yellow color mixed with the blue (due to small amounts of uranium salts and traces of picric acid), but the difference between the hydrolyzed and the unhydrolyzed sample was very striking and there is no doubt that fully half the total phosphorus present is bound, and is liberated only by acid hydrolysis. Normal blood contains, as mentioned below, approximately 20 mg. of adenine nucleotide (containing about 2 mg. of phosphorus) per 100 cc. and the amount of phosphorus liberated by acid hydrolysis in the above experiment corresponds roughly to the nucleotide content as determined by digestion and nesslerization of the uranium precipitate as outlined below.

The presence of bound phosphate in the uranium precipitate in amounts roughly corresponding to the presumptive nucleotide content would seem to be further evidence of the actual existence of adenine nucleotide.

Method.

The following method may be used for the determination of adenine nucleotide in blood.

TABLE I.
Recovery of Nucleotide Added to Blood Filtrate.

Specimen No.	Nucleotide N in 100 cc. blood.	Nucleotide N added per 100 cc.	Total amount present.	Total amount found.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	4.2	8.0	12.2	12.1
2	4.0	8.0	12.0	11.9
3	5.0	23.3	28.3	27.8

Pipette 30 cc. of the tungstic acid protein-free blood filtrate of Folin and Wu (5) into a 50 cc. pointed centrifuge tube. Add 0.5 cc. of 20 per cent uranyl nitrate solution and stir well. Centrifuge. Decant the supernatant liquid; run in another 30 cc. sample of the blood filtrate and treat this with uranium nitrate as before. Centrifuge, decant the supernatant liquid as completely as possible. Wash the precipitate twice with 5 cc. of 0.4 per cent sodium chloride in water. Now treat the precipitate with 0.5 cc. of 4 per cent sodium hydroxide and stir very thoroughly. Then add about 7 cc. of water, stir again, and centrifuge for 10 minutes. Decant the supernatant liquid into a 50 cc. digestion tube, digest, and nesslerize just as for non-protein nitrogen, according to Folin and Wu. The same standard that is ordinarily used for non-protein nitrogen generally serves here (5).

Blanks containing urea, uric acid, amino-acids, creatinine, adenine, guanine, phosphates, and all the reagents used were negative.

Normal whole blood contains, as far as our limited experiments have gone, from 15 to 25 mg. of adenine nucleotide per 100 cc. Adenine nucleotide contains approximately 20 per cent nitrogen.

TABLE II.

Specimen No	Nucleotide N per 100 cc.
	<i>mg.</i>
1	3.0
2	4.2
3	3.5
4	5.0
5	3.8

CONCLUSIONS.

Evidence is presented to show that adenine nucleotide exists in human blood in appreciable quantities, and a simple method is described whereby this portion of the undetermined nitrogen may conveniently be determined.

While it seems certain that adenine nucleotide is by far the largest representative of this class in blood, it is not improbable that nucleotides of the other purines are also present in small amounts.

It is suggested that the adenine nucleotide content of blood accounts for a considerable part of the undetermined nitrogen.

The work is being continued at the Thorndike Memorial Laboratory of the Boston City Hospital.

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A COLOR TEST FOR WATER-SOLUBLE B.

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Notwithstanding a considerable number of efforts to obtain some information concerning the chemical nature of the vitamin B, our knowledge of this substance is still very meager. Several efforts to isolate it as an individual compound have failed (1). During the progress of the operations directed toward purifying material there is invariably a loss in the physiological properties of the material under investigation.

Further experimental studies of vitamins would be greatly facilitated if it were possible to employ a chemical test for each of them. Up to the present time the only method of testing a preparation made for the purpose of isolating and identifying, or for studying the properties of a vitamin, is to feed some of the material to an animal which is confined to a diet adequate in all other respects but lacking in the vitamin in question. Judgment as to whether the substance is present or absent turns on the failure of the animal to grow or to respond with a disappearance of the symptoms characteristic of the lack of the vitamin. This method is both time-consuming and wasteful of material, and has the further disadvantage that the preparation under study may change during the interval necessary for the feeding experiment. This applies especially to experimental work involving growth with rats, guinea pigs, or other animals. I have attempted to find a specific chemical test for the vitamin B. The observations made during the pursuit of this inquiry have thrown some light on the possible nature of this substance, at least they have ruled out certain chemical types as possibly related to this substance. The plan of the present investigation may be formulated as follows:

Extracts were made with various solvents from several kinds of vegetable foodstuffs which were botanically unrelated, but which were known to contain the vitamin B. Thus, wheat embryo, navy beans, spinach, and carrots, represent a cereal germ, a legume seed, a leaf, and a fleshy root, all of which contain from moderate to abundant contents of this vitamin. It was reasoned that color reactions which might be applied to these extracts and be positive in all could provisionally be regarded as being given by the vitamin rather than by some other substance, since it is hardly to be expected that these several unrelated vegetable materials would furnish extracts containing the same list of those chemical substances present in small amounts which are more or less characteristic of individual species of plants.

As a means of controlling the study, extracts were likewise made from these same foods rich in the vitamin B, but using several solvents which have been shown by McCollum and Simmonds (2) in experiments controlled by feeding tests on the rat not to possess the power of dissolving out the vitamin B. As a further means of avoiding error in attributing positive color reactions of any type observed to the vitamin B, extracts were prepared in a similar manner to those just mentioned from polished rice, white wheat flour, and beefsteak, using both solvents which do and do not dissolve the vitamin B.

It seems certain that any color test which is always positive in any extract made from a food known to contain the vitamin B, where the preparation was made by a solvent which extracts this substance but is not given by extracts of the same foods made with solvents which do not extract the vitamin, is worthy of the most careful consideration. When, furthermore, such a test proves negative when applied to several extracts made with solvents for vitamin B from food substances of a complex nature, such as polished rice or wheat flour, which experience has shown to be essentially lacking in the vitamin B, interest in the test is greatly enhanced.

Preparation of Material for Testing.

The system of purification of the original extracts of rice polishings, wheat germ, and navy beans, was based on the method

described by McCollum and Simmonds (2); *i.e.*, of using different solvents in succession. Each stage of the purification in each case was tested with a great number of reactions.

In the case of rice polishings and wheat germ the method of preliminary purification of the extracts was the same. Finely divided material was digested for 4 days with ten times its weight of a 50 per cent water-alcohol solution at room temperature. The mixture was then heated on a water bath to incipient boiling, cooled, and the solution filtered off. The extract from 100 gm. of material was acidified with 8 cc. of 10 per cent acetic acid and again heated to boiling, cooled, and filtered. The filtrate was evaporated nearly to dryness under reduced pressure, and the residue dried over solid sodium hydroxide *in vacuo*. The residue thus obtained was extracted in a Soxhlet apparatus with ether and subsequently with absolute alcohol for 24 hours. The resulting preparation from rice polishings was dissolved in slightly acidified water and subjected to further treatment so that the following preparations resulted:

- Preparation 1....The acidified solution mentioned above.
- “ 2....Preparation 1 was alkalized and shaken out several times with chloroform.
- Preparation 3....Preparation 1 was shaken with fullers' earth, 10 gm. for each 100 gm. of rice polishings. The fullers' earth was filtered off, washed once with water, and once with alcohol.
- Preparation 4....Preparation 1 was evaporated on sand *in vacuo*, and extracted 12 hours with hot benzene.
- Preparation 5....Preparation 1 was evaporated on sand, and extracted cold with glacial acetic acid.

From wheat germ the following preparations were made.

- Preparation 6....Acidified water solution made in a manner analogous to Preparation 1.
- Preparation 7....Preparation 6 was shaken out several times with a mixture of 90 per cent chloroform and 10 per cent of 95 per cent alcohol by volume.
- Preparation 8....Preparation 6 was evaporated on sand *in vacuo* and extracted three times during 12 hours with hot benzene.
- Preparation 9....Preparation 6 was evaporated on sand *in vacuo* and extracted with cold glacial acetic acid.

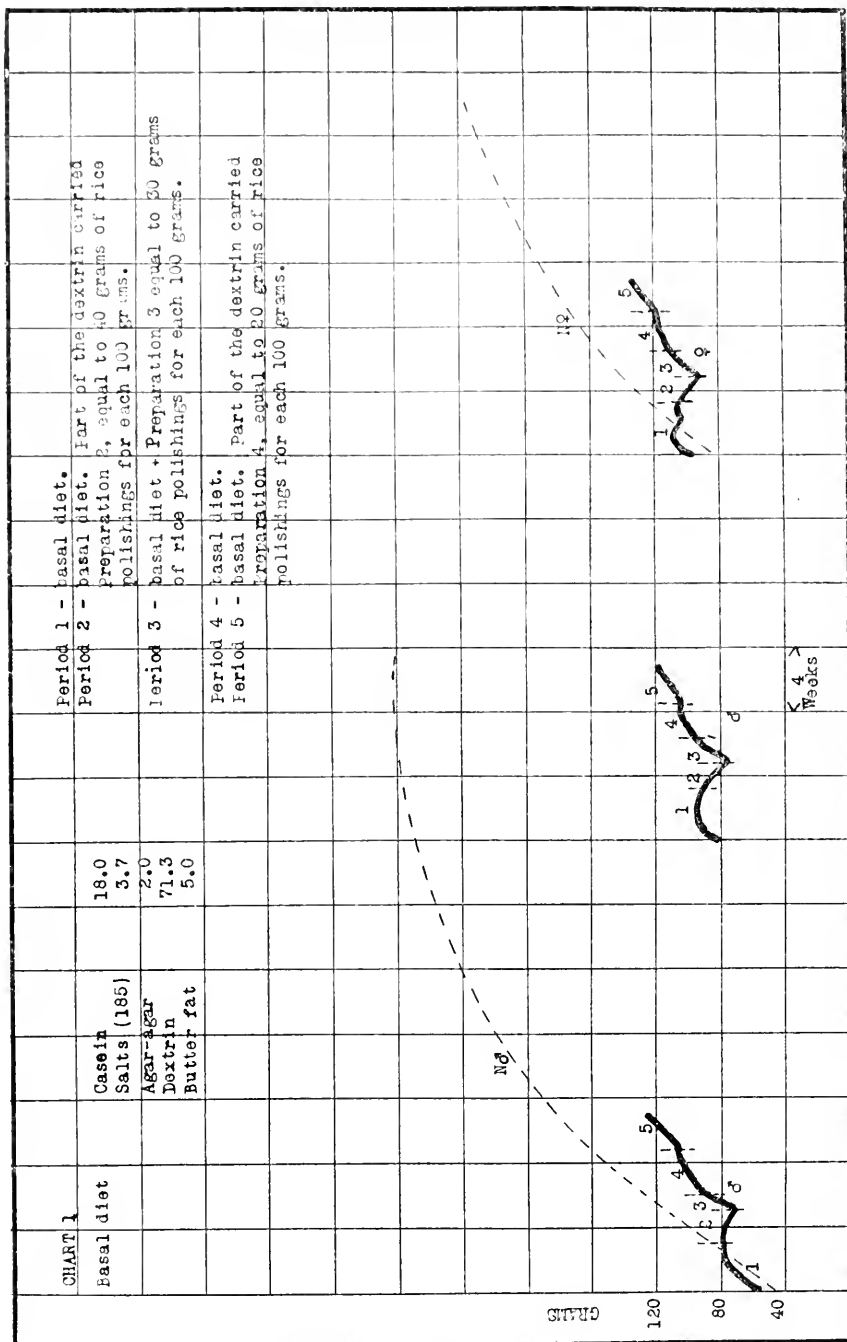


CHART 1.

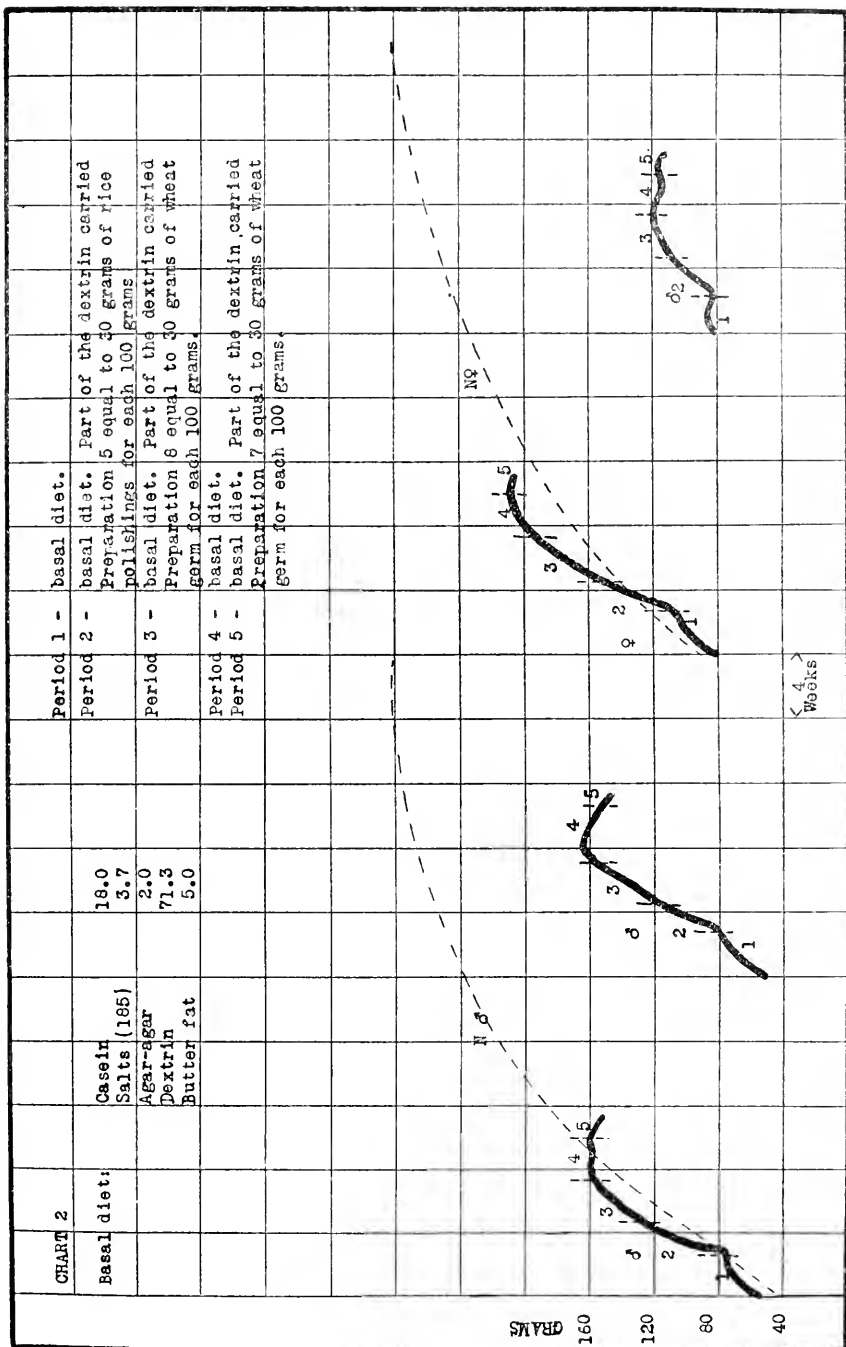


CHART 2.

The navy bean powder was subjected to another kind of treatment which was shown by McCollum and Simmonds (2) to be very efficient in extracting the vitamin B. The bean powder was extracted with ether, then with absolute alcohol, and subsequently three times for 6 hours with 90 per cent alcohol, which latter solvent extracts considerable amounts of the vitamin B.

Preparation 10. . . . The 90 per cent alcoholic extract (Preparation 9) was evaporated *in vacuo* and taken up in slightly acidified water.

Preparation 11. . . . Another portion of the 90 per cent alcoholic extract was evaporated on sand under diminished pressure, and extracted 6 hours with hot benzene.

From all the above preparations the organic solvent was distilled off *in vacuo* and the residue redissolved in water.

Extracts were prepared from tomatoes, spinach, carrots, polished rice, and white flour, using 50 per cent alcohol. These extracts were employed for various tests without further purification. An extract with 50 per cent alcohol was prepared from beef-steak (round) which had been steamed in a pressure cooker, subsequently dried, and powdered. This extract was acidified with acetic acid and boiled to precipitate foreign materials as much as possible.

Feeding tests on young rats showed that Preparations 3, 4, 5, and 8 contained the vitamin B. The results of these tests are illustrated in Chart 1, Periods 3 and 5; and Chart 2, Periods 2 and 3. These preparations were obtained from Preparations 1 and 6, which show these to be similarly active. Preparation 9 was made in a manner similar to Preparation 5, and must therefore also be active, since glacial acetic acid is known to be a good solvent for the vitamin B (3). Preparations 2 and 7 were found to be inactive (see Chart 1, Period 2; Chart 2, Period 5).

Chemical Investigation of the Preparations Described.

From the numerous chemical tests applied to these preparations the following conclusions can be drawn. The active principle (vitamin B) cannot be a phenol, since several of the active preparations failed to give phenol reactions (Millon's reaction, Liebermann's reaction, etc.).

Primary, secondary, and tertiary amine reactions were similarly negative. The highly purified preparations were free from peptones or higher protein derivatives, since the biuret reaction was negative.

With ninhydrin these preparations developed a red and not a blue color, which latter color is given by amino-acids.

Sugar tests on all active preparations were positive.

A positive test was always secured with the Folin-MacCallum reagent, but the intensity of the test diminished with increased purification.

The diazo reaction always yielded a brownish red color.

Every preparation known to contain the vitamin B gave a white precipitate with Millon's reagent.

All highly purified preparations containing little sugar after drying were soluble in concentrated sulfuric acid with a yellow color, which is about 2 hours developed into a pronounced rose color. The test was made in a porcelain dish and the rose tint appeared first on the periphery. After standing 12 hours the color disappeared and a brownish crystalline precipitate formed. The same substances dissolved in Fröhde's reagent with a brown color, which changed to a vivid green beginning at the periphery.

The color reaction described with sulfuric acid was always given by the preparations known to contain the vitamin B, but is likewise given by extracts of certain foods, made with absolute alcohol which is not a solvent for the vitamin. The test is, therefore, not specific for vitamin B.

The following test, however, has proven positive in all cases where the vitamin B was present, and always negative in preparations where it was judged to be absent, either as a result of feeding tests or because they were made with solvents which do not extract the vitamin B.

Abderhalden and coworkers (4) and Hess (5) have recently published results which indicate that tissues from different organs and muscles possess distinctly greater oxygen absorption capacity when derived from pigeons supplied with the vitamin B than do corresponding tissues from pigeons fed on polished rice as the sole food. These investigators also found that after adding solutions containing the vitamin B to the tissue preparations of polyneuritic pigeons there was an increased oxygen absorption capacity.

These observations suggested the desirability of applying a test for the vitamin B which would involve reduction. Tests could not be made in alkaline medium because of the presence of carbohydrates in the preparations, and in neutral solutions could

TABLE I.
Summary of the Results of Tests with Ferric Ferrieyanide.

Tested material.					Results.
Rice	polishings,	50	per cent	alcoholic extract.....	+
"	"			absolute alcoholic extract.....	-
"	"			ether extract.....	-
"	"			acetone extract.....	-
"	"			benzene ".....	-
Preparation	1			from rice polishings.....	+
"	2	"	"	" boiled with 5 per cent NaOH.....	-
"	3	"	"	".....	+
"	4	"	"	".....	+
"	4	"	"	" boiled with 5 per cent NaOH.....	-
"	5	"	"	".....	+
"	5	"	"	" boiled with 5 per cent NaOH.....	-
Wheat	germ,	50	per cent	alcoholic extract.....	+
"	"			absolute alcoholic extract.....	-
"	"			ether extract.....	-
Preparation	6			from wheat germ.....	+
"	6	"	"	" boiled with 5 per cent NaOH..	-
"	8	"	"	".....	+
"	9	"	"	".....	+
"	9	"	"	" boiled with 5 per cent NaOH..	-
"	10	"		navy bean.....	+
"	10	"	"	" boiled with 5 per cent NaOH...	-
"	11	"	"	".....	+
Spinach,	50	per cent		alcoholic extract.....	+
Carrot,	50	"	"	".....	+
Polished rice,	50	per cent		alcoholic extract.....	-
White flour,	50	"	"	".....	-
Beefsteak,	50	"	"	".....	-

not be relied upon to give a decisive test of this property. Accordingly I adopted as a reagent a solution of ferric chloride and potassium ferrieyanide in gram molecular ratios; *i.e.*, ferric ferrieyanide used in acid media.

Brouardel and Boutmy described in 1881 (6) the formation of a blue color by the reduction of ferric ferrieyanide as a specific

reagent for ptomaines in contrast with alkaloids. Tanret (7) and Beckurts (8) have shown that some of the alkaloids and glycosides are able to bring about the same reaction. I have tested a considerable number of different solutions containing the vitamin B (see Table I) and found these invariably to possess reducing power for the ferric ferricyanide. On the other hand, the reduction test with this reagent has invariably been negative when carried out with solutions known not to contain this active principle. Sugars and amino-acids have not produced a color change. Extracts of polished rice, white flour, and beefsteak produced a more or less vivid green color, and sometimes a precipitate of a dirty greenish color which was, however, very easy to distinguish from the blue color developed by the preparations active in that they contain the vitamin B. Solutions containing the vitamin B when boiled for a prolonged period with the addition of sodium hydroxide to make a concentration of 5 per cent no longer bring about the formation of a blue product. Instead there is formed a green color similar to that of extracts free from the vitamin B.

The color reaction for the vitamin B is carried out as follows: To the concentrated aqueous solution of the preparation in question acetic acid is added to make the concentration about 2 per cent. The reagent is freshly prepared by mixing equal volumes of tenth molar ferric chloride and potassium ferricyanide solutions. The reagent is added as long as the depth of the blue color increases. The test-tube containing the mixture is stoppered and allowed to stand 10 minutes, when the color is observed. 1 to 5 volumes of distilled water are added to reach a convenient dilution and the color is again observed. If there is not a distinct blue color, or after standing for sometime a bright blue precipitate, the test is negative.

SUMMARY.

1. A systematic series of extracts are described, some of which were made from foods containing the vitamin B, others free from that substance. The list of extracts includes those which dissolve the vitamin B, like water, dilute alcohol, and glacial acetic acid; also extracts with organic solvents which do not extract that substance.

2. Certain chemical reactions common to all the preparations containing the vitamin B are described.

3. A reduction of ferric ferrieyanide with the formation of a blue color was found to be a reaction always given by extracts containing the vitamin B.

4. In no instance was this reaction given by an extract of a food containing the vitamin B when the solvent used was one which does not extract that substance.

5. This test was in no instance given by an extract of a food which does not contain the vitamin B, although the solvents used extract this principle when present. Such solvents are water, dilute alcohol, and glacial acetic acid.

6. The plan of these experiments, involving as it did a carefully selected list of foods and solvents, strongly suggests that the substance which reduces ferric ferrieyanide under the conditions described is the vitamin B.

7. Phenols, amino-acids, and most alkaloids seem to be excluded as substances giving the reaction for this vitamin.

The work was suggested by Prof. E. V. McCollum, and the author wishes to express his thanks to him and to Miss Nina Simmonds for their advice and helpful suggestions during the course of this investigation.

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CHANGES IN THE PROTEINS AND THE GELATIFICATION OF FORMALIZED BLOOD SERUM.

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It is well known that a sufficient proportion of formaldehyde reacts with proteins to form gels. Blum (1) found that a small proportion of formaldehyde prevented the coagulation of albumins by heat and called these non-coagulable albumins "methylene-albumins." Schiff (2) and Schwarz (3) believe that the aldehyde radical combines with the NH_2 group, and thus, by converting proteins into acids, denatures them. Sørensen's (4) method of titrating amino-acids is based upon the fact that when formaldehyde in excess is added to amino-acids, the NH_2 groups are fixed as methylene groups, thus permitting the free COOH groups to be titrated. In order to insure the conversion of all the NH_2 groups to methylene groups, it is necessary to use an excess of formaldehyde, as Sørensen (4) and de Jager (5) have shown that the reaction between formaldehyde and the amino group is reversible. But in the literature on the structure and other physicochemical properties of gels, including gels of proteins with formaldehyde, no information has been found concerning the successive changes that the individual proteins undergo after treatment with formaldehyde.

While the present investigation was in progress Gaté and Papacostas (6) reported that luetic serum treated with formaldehyde gelled while non-leutic serum did not. This reaction was termed the formol-gel test and was offered as a substitute for the Wassermann test. Later, Nicolas (7) showed that this reaction was given by the normal serum of cattle and horses and also by solutions of fibrinogen. Recently, Armangué and Gonzales (8) found that serums which gave a positive reaction to this test would, if previously diluted with 6 or more parts of

water, give a negative reaction, and conversely, that serums originally yielding a negative reaction could, by concentration, be made to afford a positive reaction. From this they conclude that a positive formol reaction is not due to a specific substance in luetic serum, but to a relative increase in the normal constituents, possibly the globulins. Holborow (9) believes that the formation of gels is due to the action of formaldehyde upon serum globulins or serum albumins, or both, and considers it probable that acid proteins form gels with formaldehyde while alkali proteins do not.

TABLE I.

Changes in the Precipitation Limits of the Globulins in Formalized Hog Blood Serum.

Time of contact with formaldehyde.	Globulins.*											
	No formaldehyde.			0.09 per cent formaldehyde.			0.18 per cent formaldehyde.			0.37 per cent formaldehyde.		
	E. G.	P. G.	T. G.	E. G.	P. G.	T. G.	E. G.	P. G.	T. G.	E. G.	P. G.	T. G.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
$\frac{1}{2}$ hr.	1.32	3.08	4.40	1.37	3.16	4.53	1.43	3.13	4.56	1.68	3.20	4.88
2 wks.				2.20	3.33	5.53	3.28	2.85	6.13	4.50	2.17	6.67
4 "	1.45	3.27	4.72	3.18	2.72	5.90	4.05	2.46	6.51	Solid gel.		
6 "				4.00	2.69	6.69	4.74	2.32	7.06			
8 "	1.42	3.33	4.75	4.05	2.75	6.80	4.83					
11 "				4.08	2.70	6.78	Solid gel.					

* Results are stated in gm. per 100 cc. of serum. E.G. = euglobulins; P.G. = pseudoglobulins; T.G. = E.G. + P.G.

EXPERIMENTAL.

Banzhaf (10), Berg (11), and Homer (12) have shown that the amount of protein in serum which is precipitable by one-third saturation with ammonium sulfate is increased when the serum is heated to 60°C. for $\frac{1}{2}$ hour. In the present work it was decided to determine first whether the addition of formaldehyde to serum would cause changes in the precipitation limits of serum proteins similar to those brought about by heat.

Experiment 1.—A clear serum, obtained from healthy hogs, was divided into four lots, to one of which no formaldehyde was added, while to the

three remaining lots formaldehyde in water solution¹ was added, so that they contained 0.09, 0.18, and 0.37 per cent formaldehyde, respectively. The portion of serum to which no formaldehyde was added was frozen and kept frozen except during the time necessary for the removal of samples for analysis. The serums containing formaldehyde were kept on the laboratory table.

Samples for analysis were taken immediately after the addition of formaldehyde and subsequently at intervals of 2 weeks until the end of the experiment. The determinations of euglobulins and pseudoglobulins were made by Method 2 of Henley (13) and checked frequently by the method of Cullen and Van Slyke (14). All determinations in this and subsequent tests were made in duplicate, and the duplicates, except in a few cases, checked within 0.1 per cent. The results are shown in Table I.

In this and subsequent tables and discussions the term *euglobulin* is used to designate the material insoluble in one-third saturated solutions of ammonium sulfate (2 parts of serum to 1 part of a saturated ammonium sulfate solution), and the term *pseudoglobulin* is used to refer to the protein which is soluble in one-third saturated but insoluble in one-half saturated solutions of ammonium sulfate. The term *albumin* when used designates the protein determined by the difference between the total proteins ($N \times 6.25$) and the total globulins. Thus, the non-protein nitrogen present is shown as albumins. There is no intention of raising the question of whether the several fractions of the formalized serums are identical with similar fractions of normal serum.

The euglobulin and pseudoglobulin in the untreated frozen sample of serum remained practically unchanged during the experimental period, while in all the samples of serum to which formaldehyde was added the euglobulin greatly increased and at the same time the pseudoglobulin decreased. The speed of the reaction which results in the production of proteins insoluble in one-third saturated ammonium sulfate from the more soluble fractions appears to depend upon the concentration of formaldehyde present as shown by the time required for the euglobulins to reach a concentration of 4.00 gm. per 100 cc.; thus, 6 weeks were required for the serum containing 0.09 per cent formaldehyde, 4 weeks for that containing 0.18 per cent formaldehyde, and less than 2 weeks for that containing 0.37 per cent formaldehyde to attain that concentration of the euglobulins. The time required for gelatification to take place also varied with the

¹ The formaldehyde used in this and subsequent tests was added in the form of a solution containing 37.34 per cent formaldehyde and neutral to phenolphthalein.

concentration of formaldehyde, as is shown by the fact that the serum containing 0.37 per cent formaldehyde gelled in less than 2 weeks, while the serum containing 0.18 per cent formaldehyde gelled between the 8th and 11th week, and the serum with only 0.09 per cent never reached that condition.

The decrease in pseudoglobulins observed in Experiment 1 was wholly inadequate to account for the increase in euglobulins, for the total globulins of the formalized serum increased by 2.5 gm. per 100 cc. of the serum during the experiment.

TABLE II.

Changes in the Precipitation Limits of the Proteins of Formalized Hog Blood Serum.

Time of contact with formaldehyde.	Proteins.*								
	No formaldehyde.			0.09 per cent formaldehyde.			0.18 per cent formaldehyde.		
	E. G.	P. G.	Alb.	E. G.	P. G.	Alb.	E. G.	P. G.	Alb.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
$\frac{1}{2}$ hr.	1.27	3.43	3.27	1.56	3.33	3.08	2.78	2.34	2.85
3 wks.							4.14	2.54	1.29
5 "				3.84	1.96	2.17	Solid gel.		
8 "	1.32	3.28	3.37	3.74	2.23	2.00			

* Results are stated in gm. per 100 cc. of serum. E.G. = euglobulins; P.G. = pseudoglobulins; Alb. = albumins.

Experiment 2.—In order to determine whether albumins in the presence of formaldehyde undergo changes in their precipitation limits, the preceding test was repeated using two portions of a hog blood serum to which sufficient formaldehyde solution was added to make the formaldehyde content 0.09 and 0.18 per cent, respectively. Globulins were determined as before, and the albumins were calculated by the difference between the total protein present ($N \times 6.25$) and the total globulin found. The results are shown in Table II.

Again, the frozen serum suffered practically no change in the relative proportion of its protein constituents after 8 weeks, while in the formalized serums the albumin as well as the pseudoglobulin progressively decreased and the euglobulins showed a simultaneous and a compensatory increase.

As it was found by the preceding tests that in the serums preserved with 0.09 per cent formaldehyde euglobulins increased

in 6 weeks to about 4 per cent and thereafter remained constant, the question was presented as to whether this limitation depended upon the absolute amount of formaldehyde present or upon its concentration. This suggested a study of the reaction in diluted serums containing formaldehyde in the same concentration as in the previous tests. Furthermore, it could not be determined from the results shown in Tables I and II whether all the proteins of the serums which contained 0.18 per cent formaldehyde or more were converted to euglobulins before gelatification occurred. Had the determinations of euglobulins been made at more frequent intervals, the extent to which the

TABLE III.

Changes in the Precipitation Limits of the Proteins of Diluted, Formalized Hog Blood Serum.

Time of contact with formaldehyde.	Proteins.*											
	No formaldehyde.			0.18 per cent formaldehyde.			0.37 per cent formaldehyde.			0.74 per cent formaldehyde.		
	E. G.	P. G.	Alb.	E. G.	P. G.	Alb.	E. G.	P. G.	Alb.	E. G.	P. G.	Alb.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
$\frac{1}{2}$ hr.....	0.77	2.60	3.56	0.83	2.53	3.57	0.77	3.17	2.99	2.75	2.51	1.67
8 days.....	0.78	2.62	3.53	3.00	2.45	1.48	4.70	1.48	0.75	5.80	0.40	0.73
15 " \dagger				4.85	1.35	0.73	5.55	0.65	0.73	6.24	0.16	0.53
89 "				4.48	1.77	0.68	6.25	0.32	0.36	6.60	0.00	0.33

* Results are stated in gm. per 100 cc. of serum. E.G. = euglobulins; P.G. = pseudoglobulins; Alb. = albumins.

\dagger All samples diluted with an equal volume of 0.85 per cent NaCl solution.

transformation of proteins progressed before gelatification occurred might have been demonstrated, but, since dilution tends to prevent gelatification, it was hoped that the study of the effects of formaldehyde on the proteins of diluted serums would indicate also the extent to which the reaction might go.

Experiment 3.—To 500 cc. of fresh, clear hog blood serum were added 1,500 cc. of a physiological salt solution (0.85 per cent NaCl). This serum solution was divided into four portions, one of which was kept frozen. To the other three portions formaldehyde solution was added in such amounts that they contained 0.18, 0.37, and 0.74 per cent formaldehyde, respectively. To each of these formalized serums an equal volume of salt solution (0.85 per cent NaCl) was added on the 15th day, thus reducing the concentration

of formaldehyde by one-half. Portions of each lot were taken for examination on the 1st, 8th, 15th, and 89th days of the experiment. Unfortunately, however, the untreated serum could not be kept after the 8th day, so that no untreated control sample was available after that time. The results are shown on the basis of gm. of protein per 100 cc. of original undiluted serum.

During the course of Experiment 3, all the formaldehyde-treated serum solutions developed a slight opalescence which was more pronounced in those which contained the higher percentages of formaldehyde. The euglobulin precipitate obtained on the 8th day from the samples containing 0.37 and 0.74 per cent formaldehyde and the total globulin precipitate obtained on the 8th day from the portion containing 0.74 per cent formaldehyde formed, as soon as filtered off, opaque, white, insoluble masses similar to the white of a boiled egg. The same was true of the precipitate obtained by one-half saturation with ammonium sulfate after 15 days from the serum containing 0.37 per cent formaldehyde. Neither the one-third nor the one-half saturated ammonium sulfate precipitates from the serum containing 0.18 per cent formaldehyde ever became insoluble after separation. None of these solutions gelled even after standing for 1 year.

As may be seen in Table III, the euglobulins in the diluted serum containing 0.18 per cent formaldehyde showed a fourfold increase in the first 8 days. On the 15th day, when the increase in this fraction was sixfold, the sample was diluted so that the formaldehyde content was reduced to 0.09 per cent. After this there was no further increase in the amount of protein insoluble in one-third saturated ammonium sulfate, but, on the contrary, on the 89th day a slight decrease was indicated. This may have been due to experimental error, although the pseudoglobulins showed a slight increase at this time. The euglobulins in the samples which before dilution on the 15th day contained 0.37 and 0.74 per cent formaldehyde, respectively, increased until there was almost a complete transformation of pseudoglobulins and albumins into euglobulins. A repetition of this experiment under practically the same conditions confirmed the results obtained in Experiment 3.

Although Experiment 3 may not be regarded as exactly comparable to Experiments 1 and 2, owing to the difference in con-

centration of formaldehyde during the first 15 days of the test, yet the cessation of the reactions at that time in the sample in which the concentration of formaldehyde was reduced to 0.09 per cent points clearly to the concentration rather than to the actual amount of formaldehyde as the factor of controlling importance.

A more complete investigation of the reactions involved is at present under way.

TABLE IV.

Relation of Gelatification to Changes in Precipitation Limits of Proteins in Formalized Serum.

Time of contact with 1.45 per cent formaldehyde.	Proteins.*				
	Precipitated by saturated $(\text{NH}_4)_2\text{SO}_4$.	Precipitated by $\frac{1}{10}$ saturated $(\text{NH}_4)_2\text{SO}_4$.	Euglob- ulins.	Pseudo- globu- lins.	Albu- mins.
	gm.	gm.	gm.	gm.	gm.
Before treat- ment.....	No precipitate.	No precipitate.	0.72	2.88	3.27
2 hrs.....	" "	" "	4.66	1.90	0.31
19 "	" "	" "	6.36	0.46	0.05
26 "	" "	Precipitate.†	6.54	0.23	0.10
45 "	" "	"	6.53		
50 "	" "	4.63			
67 "	" "	5.59			
91 "	Precipitate.†	6.14			
105 "	Serum gelled.				

* Results are stated in gm. per 100 cc. of serum.

† A precipitate was obtained but could not be separated by filtration.

*Relation of Composition to Rate of Gelatification of For-
malized Serums.*

In order to determine whether the action of formaldehyde on undiluted serums would cause the production of proteins insoluble in less than one-third saturated solutions of ammonium sulfate, and also whether the extent of changes which serum proteins undergo in the presence of formaldehyde bears any relation to gelatification, the following experiment was carried out.

Experiment 4.—To a normal, undiluted, horse blood serum of known composition, formaldehyde solution in an amount sufficient to make a concentration of 1.48 per cent formaldehyde was added. Following the addi-

tion of the formaldehyde solution, the serum was examined at the intervals shown in Table IV. The amounts of euglobulins, pseudoglobulins, and albumins were determined at the 2nd, 19th, 26th, and 45th hours, and in addition, the amounts of proteins precipitated by one-tenth and one-fifth saturated ammonium sulfate were determined on the 50th, 67th, and 91st days when filterable precipitates were obtained. The results shown in Table IV are expressed as gm. per 100 cc. of serum.

These results indicate that all or practically all of the proteins of this serum became insoluble in one-fifth saturated ammonium sulfate solutions before gelatification occurred.

As already noted, Armangué and Gonzales claim that the gelatification observed by Gaté and Papacostas in certain luetic serums after the addition of formaldehyde is not due to any spe-

TABLE V.

Effect of Dilution upon Rate of Gelatification of Formalized Horse Serum.

Dilution.		Time required for 1 cc. of the serum solution to gel.			
Serum.	Water.	With 2 drops formaldehyde solution.	With 3 drops formaldehyde solution.	With 4 drops formaldehyde solution.	With 5 drops formaldehyde solution.
cc.	cc.	days	days	days	days
100	0	$\frac{3}{4}$ *	$\frac{3}{4}$	$\frac{3}{4}$	$\frac{3}{4}$
90	10	1	1	2	2
80	20	4	4	4	4
70	30	8	6	6	8
60	40	9	11	†	†
50	50	†	†	†	†

* First observation made after 18 hours, second after 24 hours, and thereafter at 24 hour intervals.

† Still fluid on the 15th day of the test.

cific property of such serums but that it depends upon the concentration of the serum proteins. The following observations bear upon this question.

Experiment 5.—A normal horse blood serum which contained 7.39 per cent total protein was diluted with varying amounts of water. Different amounts of a formaldehyde solution were added to 1 cc. portions of the various dilutions. The dilutions employed, the amount of formaldehyde solution added to each, and the time required for each diluted serum to gel are shown in Table V.

It is very apparent that the more concentrated serum solutions gelled the more rapidly. In some cases the portions con-

taining the smaller quantities of formaldehyde gelled before those of the same dilution which contained larger amounts of formaldehyde. This was perhaps due to the fact that all the formaldehyde solution present in excess of the amount required to produce gelatification served to dilute the serum and thus to retard gelatification.

TABLE VI.
Rate of Gelatification of Formalized Serums of Different Composition.

Serum.	Composition of untreated serums.*				Sublots.	Formaldehyde solution added.	Time required to gel.†
	T. P.	E. G.	P. G.	0.1 N NaOH to neutralize.			
	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>cc. per 100 cc.</i>		<i>cc. per 100 cc.</i>	<i>hrs.</i>
A	9.68	2.00	5.46	12.0	A 1	8	$\frac{1}{6}$
					A 2	4	$\frac{1}{5}$
					A 3	2	3
B	7.62	0.94	4.38	2.0	B 1	8	11
					B 2	4	23
					B 3	2	47
C	7.37	1.12	2.67	Alkaline.	C 1	8	29
					C 2	4	47
					C 3	2	72
D	7.19	0.93	3.10	3.0	D 1	8	23
					D 2	4	47
					D 3	2	96
E	6.18	0.56	2.64	4.0	E 1	8	All fluid
					E 2	4	on the
					E 3	2	13th day.

* T.P. = total proteins ($N \times 6.25$); E.G. = euglobulins; P.G. = pseudoglobulins.

† Observations were made at frequent intervals during the day and early evening.

Only one serum was used for the varying dilutions of Experiment 5. In order to determine to what extent gelatification is dependent upon variations in the individual serum proteins apart from mere dilution, the rate of gelatification of five different formaldehyde-treated serums of known composition was determined.

Experiment 6.—The blood serums from five different normal hogs were analyzed for total protein, euglobulin, pseudoglobulin, albumin, and titratable acid, using phenolphthalein as an indicator. Portions of 25 cc. of each serum were treated with 0.5, 1.0, and 2.0 cc. of formaldehyde solution, respectively, and were then kept on the laboratory table in stoppered bottles until gelatification occurred, the time required being noted in each case. The last four serums were examined simultaneously, while the first was studied in the same way at an earlier date. The results are given in Table VI.

No relation is apparent between the titratable acid of these serums and the time required for gelatification. Thus, Serum C was alkaline to phenolphthalein and Serum D was acid; both contained approximately the same amounts of proteins, and both gelled in approximately the same time.

It is perfectly apparent that the rate of gelatification is proportional to the concentration of total proteins in these serums. But the failure of Serum E, which contained 6.18 per cent total protein, to gel in 13 days indicated that the protein concentration could not be the sole factor controlling the rate of gelatification, for serums containing even less protein than Serum E have been found to gel in less than 13 days under similar conditions. In Experiment 5 the rate of gelatification was found to depend upon the concentration of the serum constituents. In that experiment water had been used as the diluent, so the different dilutions tested varied not only in concentration of proteins but also in concentration of salts. In order to determine the effect of varying the protein concentration while holding the salt concentration approximately constant, the following experiment was made.

Experiment 7.—Portions of a normal horse blood serum, which contained 7.14 per cent total proteins, were diluted with varying amounts of water, and other similar portions were diluted with correspondingly varying amounts of an 0.9 per cent NaCl solution. Portions of a normal hog blood serum, which contained 7.0 per cent total protein, were similarly diluted. To 1 cc. portions of the varying dilutions of each serum different amounts of a formaldehyde solution were added. The dilutions employed, the amounts of formaldehyde added, and the time, expressed in hours, for gelatification to take place are shown in Table VII. Observations were made at frequent intervals between 7 a.m. and 10 p.m.

Again, the undiluted serums all gelled in less time than did the diluted ones. Of more importance is the fact that the serums

diluted with salt solution gelled, in all cases in which differences are shown, before the same serums diluted with corresponding amounts of water. In a few instances the serums diluted with corresponding amounts of water and salt solution are shown as having gelled at the same time. This is explained by the fact that these serums gelled between 10 p.m. and 7 a.m., hours during which observations were not made.

In this connection the following observation is interesting: It will be recalled that none of the formalized samples of Serum E, Experiment 6, had gelled on the 13th day. On that day one

TABLE VII.

Effect of Salt and Protein upon Rate of Gelatification of Formalized Serums.

Kind of serum.	Dilution.		Kind of diluent.	Time required for 1 cc. of the serum solution to gel.				
	Serum.	Diluent.		With 1 drop formaldehyde solution.	With 2 drops formaldehyde solution.	With 3 drops formaldehyde solution.	With 4 drops formaldehyde solution.	With 5 drops formaldehyde solution.
	cc.	cc.		hrs.	hrs.	hrs.	hrs.	hrs.
Horse.	100	0	None.	57	50	44	44	44
	90	10	Salt solution.	151	132	92	77	92
	90	10	Water.	*	144	144	132	106
Hog.	100	0	None.	75	42	23	20	17
	90	10	Salt solution.	138	66	42	42	42
	90	10	Water.	192	70	45	42	42
	80	20	Salt solution.	240	138	78	66	48
	80	20	Water.	*	216	162	116	112

* Fluid after 15 days.

of the samples was divided into two portions of 12.5 cc. each, to one of which 0.5 gm. of NaCl was added, while the other portion was left untreated. On the next day, the 14th day of the test, the portion to which the additional salt was added had gelled, while the portion to which no salt was added was still fluid on the 13th day thereafter, or the 27th day of the test.

From the above it is apparent that the rate of gelatification of serums treated with given amounts of formaldehyde is proportional to the concentration of salts as well as to the concentration of total protein, these two factors being perhaps of about

equal importance, at least within the limits of concentration which obtained in these experiments.

SUMMARY.

1. The addition of formaldehyde to blood serum causes a progressive decrease in the solubility of the proteins in ammonium sulfate solutions. With respect to solubility in that reagent the albumin behaves as if changed to pseudoglobulin, the pseudoglobulin as if changed to euglobulin, and the euglobulin, in its turn, as if changed to protein of still lower solubility.

2. With a given serum the rate of transformation is proportional to the concentration of formaldehyde and may proceed to the complete disappearance of the albumin and pseudoglobulin fractions. If, however, the concentration of the formaldehyde is sufficiently low, this transformation appears to reach an equilibrium, independent of the concentration of the proteins, before it is complete.

3. With a given undiluted serum the rate of gelatification is proportional to the concentration of formaldehyde. Dilution of a serum beyond certain limits, of course, prevents gelatification.

4. With a given concentration of formaldehyde, the rate of gelatification is proportional to (a) the concentration of the protein, and (b) the concentration of the salts, both factors appearing to be of about equal importance within the range studied.

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EXTRACTION OF VITAMINS FROM YEAST AND RICE POLISHINGS WITH VARIOUS WATER-MISCIBLE SOLVENTS.*

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For the chemical study of the vitamins in yeast, rice polishings, or any other material, the selection of a suitable solvent is of paramount importance. While we encounter in the literature some isolated data on this subject, no systematic study involving the comparative values of a number of liquid solvents has so far been undertaken. Water, alcohol, and acidulated alcohol have been used here and there. Ether has never found favor, because even early in vitamin research, workers had convinced themselves that this liquid extracts at best but traces of vitamin B and associated substances. One of the present authors showed as early as 1912 (1) that 95 per cent alcohol was a very poor solvent for vitamin B; even repeated treatments with boiling alcohol of this concentration failed to diminish the activity of the residue to any extent, though yielding potent extracts.

McCollum and Simmonds have conducted experiments on the extraction of vitamin B from beans, pig liver, and the wheat germ (2). In a recent paper Levine, McCollum, and Simmonds (3) recommend glacial acetic acid as a solvent for vitamin B in navy beans. They do not, however, state the final dilution of the acetic acid after it has been mixed with the beans (which, of course, contain different amounts of moisture), nor do they state whether any tests were carried out with the residues as well as with the extracts—a very important point.

The ideal solvent for vitamin B would be one which extracts it quantitatively without extracting any other vitamin or any inert

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material. The guiding idea, then, in our work, was to ascertain which among a number of solvents would give a potent extract and at the same time carry over a minimum amount of substances that accompany the vitamins—a potent extract with the least possible amount of impurities. This, in our minds, is an essential preliminary step in the attempted isolation of a vitamin.

For some time past one of the authors has used a method of extraction that he has found quite serviceable. If one suspends pressed yeast (which usually contains in the neighborhood of 70 per cent moisture) in enough 95 per cent alcohol so that the resulting solution is 70 per cent in strength, filters the mixture, and repeats this treatment on the residue two or three times, one is able, as a rule, to obtain a very active extract and a quite inactive residue. We have amplified this procedure in the present instance, using a number of different solvents, the selection of these being dependent upon their miscibility with water and their low boiling point. The encouraging results with 70 per cent alcohol also led us to several experiments with different concentrations of this solvent. Lastly, in the hope that rice polishings might prove a more desirable "raw" material than yeast, a number of extraction experiments were carried out with them.

The possibility that a single solvent may have a selective dissolving power for a particular vitamin or vitamin-like substance (either vitamin B or D) led us to extend our field of investigation. We not only tested the extracts *and the residues* on pigeons but also on rats. We further investigated the extracts with regard to their yeast growth-promoting power (due to vitamin D) and their content of coenzyme.

EXPERIMENTAL.

Method of Extraction.—Brewery yeast¹ was freed from as much liquid as possible by pressure in a tincture press, and the percentage of moisture in the residue determined. This averaged about 70 per cent. Tests were next made with the material to determine its vitamin content. 0.33 gm. of test material per pigeon per day was sufficient to maintain the birds in good health. To 300 gm. of the pressed yeast enough of the solvent was added to give a final concentration of the desired strength. The mixture was kept in a stoppered bottle for 48 hours, shaken from time to

¹ We wish to thank Ruppert and Co., New York, for the yeast used in these experiments.

time, filtered, and the residue triturated with 200 cc. of fresh solvent.² This was filtered, and the operation repeated twice. The final washings were made with 100 cc. of solvent.

The filtrate and washings were evaporated *in vacuo* to 300 cc. (so that 1 cc. of the solution corresponded to 1 gm. of the pressed yeast), and the solution was kept in a stoppered bottle in an ice chest. Portions were used for the determination of total solids and of total nitrogen, yeast growth-promoting power, and coferment activity. The rest was used in the tests on rats and pigeons.

With rice polishings the procedure was somewhat different. To 500 gm. to rice polishings³ (containing about 8 per cent of moisture) 2,500 cc. of solvent of the required strength were added. The subsequent procedure was similar to that described under yeast except: (a) after filtration, the residue was triturated with 330 cc. of solvent, and this was repeated twice, and finally once with 160 cc.; and (b) the filtrate and washings were evaporated to 250 cc. (although in one or two cases, owing to the presence of large quantities of dissolved material, the evaporation could not conveniently be carried below 350 cc.). Extracts from rice polishings rich in fat-like substances were difficult to filter clear.

Animal Experiments.—For these experiments twenty-four pigeons and eighteen rats were used. The pigeons were kept in individual cages, described in an earlier paper (4). Their weight and intake of polished rice were recorded daily. Every morning after weighing, each pigeon was given the preparation to be tested. All the tests lasted a week—a length of time found by previous experience to be sufficient. In some instances the period was lengthened to confirm results. A group of six birds was used for testing each extract and residue and the averages were taken. The group of pigeons which received an inactive residue or poor extract 1 week was given the following week a preparation which we had reasons to believe might prove active.

The eighteen young rats used for similar tests were also kept in separate cages. Their diet was the usual food free from vitamin B; according to the Osborne-Mendel formula:

	<i>per cent</i>
Casein.....	18
Corn-starch.....	54
Butter.....	18
Lard.....	6
Salt mixture.....	4

² If the original concentration was 70 per cent, then the solvent added at this point was 70 per cent also.

³ We wish to thank Mr. Charles E. Armstrong, 44 Whitehall St., New York, for his kind assistance in securing the rice polishings.

The fractions to be tested were given daily in individual cups. If any was left, as in the case of some of the residues, it was mixed with the food to insure its being taken. The weight and food intake were recorded twice a week. Each test lasted 2 weeks. The averages of groups of three were taken for each test on an extract or residue, and the groups on extracts and residue were alternated.

Yeast Growth.—One of us (5) has shown that the factor which induces yeast growth is a fourth vitamin, which we have named vitamin D (identical, it would seem, with the "bios" of Wildiers). This opinion has recently been confirmed through work in the Biochemical laboratory at Oxford (6). The method adopted for measuring yeast growth was that devised by one of us working in conjunction with Dubin (7).

Coferment.—Harden, Thompson, and Young (8) have described a method for determining the coenzyme, and we refer readers to their paper for details. For the test a culture of yeast was grown in Nägeli solution (containing 5 per cent autolyzed yeast as a source of vitamin D). After 48 hours the solution was centrifuged and the cells were washed five times to remove all traces of coferment. A uniform suspension was made from the centrifuged cells, and divided between four flasks, two to act as controls, and two others for the extract to be investigated. The contents of the "control" and "test" solution were as follows:

Cane-sugar	5 gm.
Extract to be tested	2 cc.
Or water in controls.....	2 "
Yeast suspension.....	10 "
2 per cent sodium dihydrogen phosphate.....	5 "
Total volume.....	50 "

Fermentation tests were made according to the method of Tholin (9), published from Euler's laboratory. The flasks were saturated with CO₂. The amount of gas recorded in Table I represents the difference between the amount of gas evolved in the experimental flasks and the controls in the course of 2 hours.

Tables.—The results have been tabulated in Table I. The figures for "nitrogen" and "solids" represent percentages calculated to a dry basis. Experience has shown that positive re-

TABLE I.

Solvent.	N	Solids.	Extract fed to pigeons.		Extract fed to rats.		Residue fed to pigeons.	Residue fed to rats.	Yeast growth (0.5 cc. extract).	Coferment (2 cc. solution). (Amount of C_{12} in 2 hrs.)
			No. of days.	Average amount per day.	No. of days.	Average amount per day.				
Yeast.										
	per cent	per cent		gm.		gm.	gm.	gm.	mm.	cc.
Methyl alcohol, 60 per cent.....	1.36	5.31	8	+0.67	10	+1.05	-0.5	+0.15	8.75	6
“ “ 70 “ “.....	1.32	5.52	8	+1.23	12	+0.71	-0.05	+1.23	7.75	15
Ethyl “ 50 “ “.....	1.14	4.30	8	+0.04	14	+0.4	-0.5	-0.64	8.0	43
“ “ 60 “ “.....	1.03	4.05	8	+0.46	14	+1.33	-0.53	+0.8	11.5	14.2
“ “ 70 “ “.....	1.07	4.16	9	+1.28	14	+1.57	-0.7	+0.80	11.0	8.2
“ “ 80 “ “.....	0.81	3.10	8	+0.3	14	+0.11	-0.6	+0.66	6.0	4.7
Propyl “ 70 “ “.....	0.73	4.09	8	+0.85	14	+2.27	-0.7	+0.58	10.0	7
Butyl “ 70 “ “.....	0.07	0.55	7	-1.3	14	-0.02	+1.2	+3.0	3.5	10
Isobutyl “ 70 “ “.....	0.22	1.0	8	-0.75	14	-0.23	+0.8	+1.47	2.5	11
Acetone, 70 per cent.....	0.22	2.07	8	+1.22	14	+2.61	+0.12	+0.73	8.0	12
Methylethyl ketone, 70 per cent ..	0.14	2.05	8	-0.8	14	-0.32	+0.77	+2.23	3.0	0
Acetic acid, 70 per cent.....	0.81		8	+0.57	14	+0.5	-1.02	+0.28	13.0	16.7
Rice polishings.										
Ethyl alcohol, 50 per cent.....	0.15	21.53	8	+0.57	14	+2.2	-0.58	+0.08	7.0	0
“ “ 60 “ “.....	0.05	12.31	8	+0.18	14	+1.4	-0.86	-1.3	2.0	5.25
“ “ 70 “ “.....	0.17	52.46	8	+0.16	14	+4.6	-0.52	+1.7	6.5	18.5
“ “ (hot), 70 per cent.....	0.11	20.91	7	+0.23	14	+0.65	-0.85	0	9.3	0
Propyl “ 70 per cent.....	0.060	19.00	7	-0.48	14	+2.6	-0.26	+1.8		
Acetone, 70 per cent.....	0.16	18.32	7	+0.014	14	+2.8	-0.65	+1.8		

sponses may be expected in less than a week in pigeons and in less than 2 weeks in rats. The plus and minus figures represent the percentage increase or decrease in weight. As, owing to unavoidable circumstances, the duration of the experiments was not always

TABLE II.

Solvent.	Calculated to N = 1.	
	Extract fed to pigeons.	Extract fed to rats.
Yeast.		
Methyl alcohol, 60 per cent.....	+0.50	+ 0.80
“ “ 70 “ “	+0.93	+ 0.53
Ethyl “ 50 “ “	+0.03	+ 0.3
“ “ 60 “ “	+0.44	+ 1.33
“ “ 70 “ “	+1.2	+ 1.4
“ “ 80 “ “	+0.37	+ 0.13
Propyl “ 70 “ “	+1.1	+ 3.1
Butyl “ 70 “ “	-0.09	- 0.009
Isobutyl “ 70 “ “	-0.17	- 0.5
Acetone, 70 per cent.....	+5.5	+11.8
Methylethyl ketone, 70 per cent.....	-0.1	- 0.04
Acetic acid, 70 per cent.....	+0.7	+ 0.6
Rice polishings.		
Ethyl alcohol, 50 per cent.....	+3.8	+14.7
“ “ 60 “ “	+3.6	+28
“ “ 70 “ “	+0.94	+27.0
“ “ (hot), 70 per cent.....	+2.0	+ 6.0
Propyl “ 70 per cent.....	-0.03	+43.3
Acetone, 70 per cent.....	-0.002	+17.5

uniform, the figures actually given represent the percentage increase (or decrease) divided by the number of days of the experiment—in other words, the *average* percentage increase or decrease *per day*. This also applies to Columns 5 and 6.

Table II has been prepared to emphasize the *comparative* features of the experiments. We have taken as our standard 1 per cent of nitrogen as a unit, and Columns 1 and 2 are really Columns 4 and 6 of Table I recalculated on the basis of N=1. This table seems to us of value in answering the question, which

solvent extracts a maximum of the vitamin and a minimum of the nitrogenous impurities?⁴

DISCUSSION.

Let us first confine our attention to the action of the various solvents on yeast. If in judging activity the standard selected is not merely one that yields an active extract, but also one that leaves behind an inactive residue, then we must select 70 per cent alcohol as the one among the solvents that most nearly approaches this standard. If, on the other hand, our guide is not so much the inactivity of the residue as the relatively small amounts of nitrogenous impurities that accompany the extract, then we would be forced to select acetone as the best among the solvents (see more particularly Table II, which was primarily prepared to emphasize this point).

On the whole it may be said that the nitrogen content of the extract is a pretty good criterion of its vitamin activity, for, as a rule, the higher the percentage of nitrogen in the extract, the more active it is apt to be. It might also be pointed out in this connection that the percentage of nitrogen and the total solids in the extract run fairly parallel.

Somewhat less definite, though fairly well marked, is the tendency for vitamin D (as measured by yeast growth) and vitamin B (as measured in pigeons) to run hand in hand, for usually the more potent the extract is on pigeons, the higher is its vitamin D content. When, however, we come to the coferment, there seems to be little relation between it and vitamins B and D, which perhaps suggests that the coferment is in no way related to these vitamins.

The hope that a single solvent would show a selective affinity for either vitamin B or vitamin D, or that a particular solvent

⁴ One feature of the table must be made clear. Under butyl alcohol, the figure is *negative*, with an extract containing 0.07 per cent N which causes a percentage loss of weight in pigeons of 1.3 (−1.3). Were the extract to contain 1 per cent N, it cannot be assumed that the figure −1.3 will be *increased*, but rather that it will be *decreased*. Hence in such cases we have arbitrarily divided rather than multiplied. (In one particular example, $\frac{1}{0.07} = 14.3$ and $\frac{-1.3}{14.3} = -0.09$.)

would yield an extract that would induce growth in pigeons and not in rats (or perhaps *vice versa*) has not been realized. But there is one point that is worthy of attention. A glance at Table I will show that every extract that showed itself active for pigeons was also active for rats. This, however, is not true of the residues, for here a number exert positive influence upon rats, and a negative one upon pigeons. The reverse is not true: in no case is there a positive response from pigeons that is not accompanied by a positive one from rats. This permits of a number of speculations. May we, perhaps, assume that the vitamin for rats is less easily soluble in the solvents used than the vitamin for pigeons?

When we examine the results with rice polishings, we find that they are not wholly comparable to those with yeast. Here 60 per cent alcohol seems to be a better solvent than 70 per cent, for not only is the percentage of N in the 60 per cent extract less than in the 70 per cent, but distinctly inactive residues are obtained. By comparison with either of these two, hot 70 per cent alcohol (heating the polishings and solvent in a water bath under a reflux condenser) is poor, contrary to what one might expect, though good for vitamin D.

The encouraging results with acetone and propyl alcohol when these were used in extracting vitamin from yeast (see Table II), led us to apply these solvents in extracting the vitamin from rice polishings. The results were disappointing, where the pigeons were concerned, but the rats fared quite well. And at this point we are led to comment on the fact that the extracts from rice polishings obtained with different solvents were uniformly better for rats than for pigeons. An extreme example of this difference is seen in the case of propyl alcohol (see Table II). May we assume that extracts of the rice polishings are particularly well supplied with the vitamin needed by rats? Must we reconsider the entire question of nutritional needs and declare, with McCollum, that the seed germ is a much more satisfactory vitamin source for rats than is yeast?

Apparently the coferment activity of the various extracts depends little, if at all, upon the amounts of vitamins B and D present. We may, however, be allowed to point out two suggestive facts: (1) that the butyl and isobutyl extracts of yeast were

rich in coferment, though they quite lacked vitamins B and D; and (2) that there was complete absence of coferment in the methylethyl ketone extract of yeast, and in the 50 and 70 (hot) per cent alcoholic extracts of rice polishings. The various tests were carried out independently by the authors and final notes compared in preparing the tables. We wish to regard these preliminary studies not so much in the nature of settling differences, as suggesting further work.⁵

SUMMARY.

1. The comparative values of the following solvents in extracting vitamin from yeast were studied: ethyl alcohol (50, 60, 70, and 80 per cent), methyl alcohol (60 and 70 per cent), propyl alcohol (70 per cent), butyl alcohol (70 per cent), isobutyl alcohol (70 per cent), acetone (70 per cent), methylethyl ketone (70 per cent), and acetic acid (70 per cent). The extracts and residues were tested on pigeons and rats; and the extracts were also tested for their content of vitamin D, coferment, total N, and total solids.

2. If inactivity of residue be taken as a criterion, then 70 per cent alcohol has proved to be the best solvent for vitamin among the solvents used.

3. If, however, we stress not only the high degree of activity of the extract, but also the low proportion of nitrogenous and other impurities (selecting the one that gives a minimum of such impurities) accompanying the vitamin, then the best among the solvents is acetone.

4. On the whole, the higher the N content of the extract, the greater the percentage of total solids, and the greater the activity of the extract.

5. Vitamins B and D tend to run parallel with one another, so that, as a rule, the higher the content of vitamin B, usually the higher is that of vitamin D.

6. The coferment shows no definite relation to either vitamin B or D.

7. Using rice polishings in the place of yeast, we find that 60 per cent alcohol is better than 70 per cent.

⁵ We wish to thank Professor W. J. Gies for his cooperation.

S. The extracts from rice polishings seem to be particularly active when tested on rats, and by comparison, far less so when tested on pigeons.

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STUDIES OF AUTOLYSIS.

IX. HYDROGEN ION CONCENTRATION IN AUTOLYSIS.

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All studies of autolysis which have concerned themselves with effect of reaction agree that acidity increases both the rate and the extent of the process (1). Arinkin (2) was the first to compare the effect of various acids in equivalent amounts and found that all accelerated autolysis. The strong mineral acids required less to give the maximum digestion, however, than the weaker organic acids. This suggests that the H ion concentration is a determining factor. On the other hand, the observations of Yoshimoto (3) on the very large increase of autolysis produced by such weak acids as boric, salicylic, and carbonic, seem to indicate that the quantity of acid rather than the H ion level plays the important and determining part in the process since these acids are too weak to raise materially the H ion level of a tissue *brei* already definitely acid from its own metabolites. Many of our own observations have suggested that the amount of the acid irrespective of its degree of dissociation was at least one determining factor in the mechanism which controls the speed and extent of tissue atrophy.

In this paper we have collected material obtained from time to time in connection with our studies of autolysis bearing upon this relation between acid, H ion, and extent of amino-acid production.

H Ion Changes at Death.

At the moment preceding death it seems fair to assume that the H ion concentration within the cell is not far from that without. With the blood at a pH=7.4 we assume the pH of the cell

as 7.2—based on considerations of the Donnan equilibrium (4) and the known difference in the pH between the corpuscles and the plasma. Immediately following death the reaction of the cells, as measured in the hashed material, changes with almost explosive rapidity. The circumstances of death appear to influence this change and the metabolic condition of the liver itself may very probably also determine the rate and extent of acid production. In a subsequent paper we shall present in more detail the data substantiating these assumptions. Suffice it to say here that the H ion concentration rises so rapidly that within 4 minutes of the death of the animal as a whole, the liver brei will have a pH of about 6.8 or less. Within a few hours the bulk of acidity has been produced and thereafter the rise in H ion is slow, reaching a maximum in 1 or 2 days. Individual livers differ markedly in the absolute figures obtained, but in a general way the curves

TABLE I.
Pig Liver.

Time	Hrs.	Days.								
	5	1	2	3	4	5	6	10	21	
pH.....	6.63	6.48	6.48	6.50	6.55	6.58	6.62	6.66	6.50	
0.2 N amino-acid, cc.....	0.30	1.15			1.55			1.90	2.20	

of change are characteristic. Thus, in one instance a liver brei gave a pH of 5.64 within 4 hours of the death of the animal and showed no subsequent increase. More often the pH attained in 4 hours is about 6.7—and the maximum acidity 6.4 is attained in 24 to 48 hours. In Table I and Fig. 1 the changes that have been observed in a typical case are presented.

It will be seen that the initial production of acid is extremely rapid after death. Within a very few minutes, or perhaps seconds, the H ion concentration has risen to the point at which the primary protease of the liver is active, and at which the base-protein salts of the living tissue are changing to acid-protein salts. At this point autolysis begins. If this conception of the process is substantially correct there should be no appreciable time interval between death of a cell or tissue and the initiation of the autolytic mechanism. Our previous study of the so called "latent period" in autolysis (5) confirms this point. If products

of initial cleavage are taken as the criterion of the start of autolysis, no latent period can be demonstrated. The appearance of amino-acids, however, is much later, as might be expected since they represent the last step in a series of cleavages (6).

The initial explosive production of acid at death is followed by a slower increase reaching the maximum in a few hours to 2 days. The amino-acids appear after a latent period of about 2 hours, and continue to increase for several days. After the initiation of the process by acid production and primary cleavage of the acid-protein salts, the process goes on to completion regardless of subsequent changes in the H ion concentration (7, 8).

The addition of glucose to a liver brei is believed to check the autolytic mechanism by inhibiting the enzymes (9). Shaffer

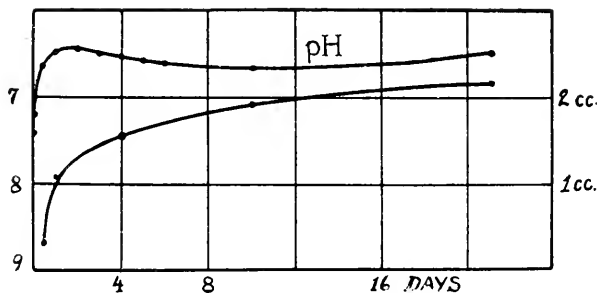


FIG. 1. pH and amino-acids.

explains the protein-sparing action of carbohydrate food in conditions like fever or starvation upon this property of glucose. On the other hand, if glucose does inhibit autolysis we should expect it to be due to preventing the regular rise of the H ion concentration. We have therefore added glucose to autolyzing liver and measured both the H ion concentrations and the rate of autolysis. Our results tabulated in Table II do not confirm Shaffer's observations. Glucose appears to increase the H ion very slightly when added to a control, an alkaline, or an acid brei. Its effect on autolysis was, however, too small to measure.

Oxygenation appears to increase slightly the acid production in an alkaline brei over the control, but the addition of glucose to such an alkaline oxygenated brei does not increase the acid production. See Table III.

The failure of glucose, in its stable form, to modify in either sense the autolysis of a tissue does not wholly rule out the possibility that glucose freshly liberated from its combinations may

TABLE II.

*Pig Liver.**

No.	Condition.		Days.					
			0	1	3	5	9	16
I	Control.....	Amino-acid..	0.40	1.15	1.25	1.45		
		pH.....	6.60	6.51	6.52		6.53	6.38
II	" + 12½ cc. 0.2 N NaOH.....	Amino-acid..	0.40	0.75	0.85	0.95		
		pH.....	7.77	7.14	7.05		6.96	6.87
III	Control + 25 cc. 0.2 N NaOH.....	Amino-acid..	0.40	0.60	0.65	0.70		
		pH.....	9.17	7.96	7.73		7.50	7.38
IV	Control + 12½ cc. 0.2 N HCl.....	Amino-acid..	0.40	1.85	2.80	3.10		
		pH.....	5.39	5.60				
V	Control + 10 gm. glu- cose.....	Amino-acid..	0.40	1.20	1.25	1.45		
		pH.....	6.57	6.44	6.49		6.53	6.35
VI	Control+10 gm. glucose + 12½ cc. 0.2 N NaOH.	Amino-acid..	0.40	0.70	0.80	0.95		
		pH.....	7.65	7.03	7.02		6.93	6.86
VII	Control+10 gm. glucose + 25 cc. 0.2N NaOH.	Amino-acid..	0.40	0.60	0.70	0.70		
		pH.....	8.93	7.93	7.58		7.44	7.35
VIII	Control+10 gm. glucose + 12½ cc. 0.2 N HCl.	Amino-acid..	0.40	1.85	2.80	3.15		
		pH.....	5.24	5.35				

* Except where otherwise specified in this and subsequent tables time is indicated in days, and amino-acid as cc. of 0.2 N in 25 cc. of the trichloroacetic acid filtrates which represent 6.25 cc. of the original brei.

have such an effect. It is possible that the unstable modification of glucose as first liberated from glycogen is more easily oxidized by the tissues, or is otherwise more reactive. As a rough test of this possibility glycogen and starch were added to liver brei and the effect on autolysis was determined. The results appear in Table IV.

TABLE III.
pH Values of Pig Liver.

No.	Condition.	Day.		Difference.
		0	1	
I	Liver (50 gm.) + 50 cc. 0.1 N NaOH.....	9.27	7.93	1.35
II	" (50 ") + 50 " 0.1 " " oxygenated.	9.44	7.66	1.78
III	Same as No. II + 5 gm. glucose oxygenated....	9.30	7.67	1.64

TABLE IV.
Pig Liver.

No.	Condition.	Days.				
		0	1	4	8	23
I	Control.....	0.50	1.60	2.00	2.25	2.90
II	" + 5 gm. glycogen.....	0.50	1.50	2.00	2.20	2.80
III	" + 5 " starch (boiled) ..	0.50	1.50	1.80	2.15	2.75
IV	" + 5 " soluble starch ..	0.50	1.65	2.00	2.30	3.00

H Ion Changes in Tissue Made Acid or Alkaline.

In the course of our studies of autolysis under artificially controlled reactions observations have been made on the effect of added acid and alkali. A strong acid like HCl increases autolysis up to the point of optimum digestion. Beyond this point further addition of acid inhibits the process. If a weak acid is added, such as acetic, the same optimum point is reached as with HCl, but the phenomenon of inhibition does not appear. It is evident, therefore, that the H ion concentration attained in the strong acid-liver mixtures produces the inhibition, while the H ion level attained with weaker acids is too low to produce this effect. Evidence has been presented that this is inhibition of the initial primary cleavage rather than the secondary or ereptic cleavage (8).

TABLE V.
Pig Liver.

No.	Condition.		Days.									
			0	1	2	3	4	5	6	10	21	
I	Control.....	Amino-acid... pH.....	0.30 6.76	1.15 6.44	6.44	6.50	1.55 6.53	6.60	6.64	1.90 6.67	6.49	
II	" + 12½ cc. 0.2 N HCl.....	Amino-acid... pH.....	0.30 5.27	1.70 5.58	5.67	5.69	3.00 5.71	5.74	5.76	3.55 5.83	5.98	
III	" + 25 " 0.2 " ".....	Amino-acid... pH.....	0.30 4.63	1.65 4.83	4.83	4.78	3.25 4.94	4.86	4.88	3.80 4.87	4.84	
IV	" + 12½ " 0.2 " NaOH.....	Amino-acid... pH.....	0.30 7.89	0.60 7.05	7.08	7.02	0.80 7.02	7.00	6.99	0.90 6.98	6.88	
V	" + 25 " 0.2 " ".....	Amino-acid... pH.....	0.30 9.45	0.55 7.87	7.68	7.55	0.70 7.47	7.44	7.43	0.80 7.32	7.27	

When alkali is added to a liver brei it produces a characteristic and sharp inhibition. If a small amount of alkali is added the mixture will in time become neutral or even acid to litmus, and a small digestion of the proteins takes place. With larger amounts the inhibition is practically complete. The same sharp checking of the autolytic mechanism is observed when some insoluble oxide or carbonate is added which will prevent the development of acidity.

In Table V and Fig. 2 the changes in H ion concentration in a typical series of modified tissue breis are illustrated.

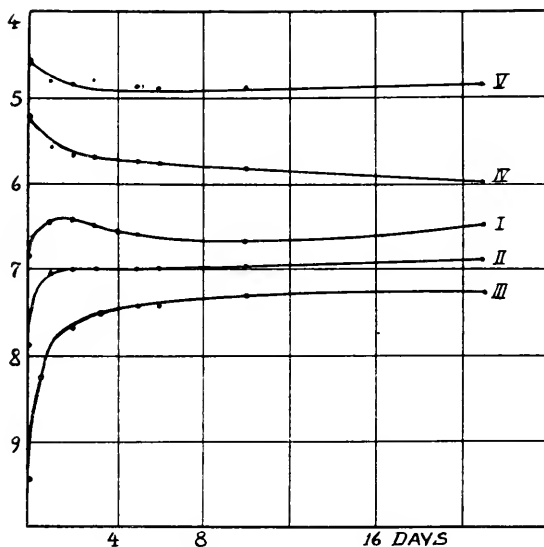


FIG. 2. pH changes in autolysis.

The outstanding features in the experiment are: (a) the small increased buffering of the acid digests where the protein is most quickly hydrolyzed into its amino-acids; (b) the very large buffer effect found in the more alkaline mixtures where there is no proteolysis at all; and (c) the tendency of all the digests to converge toward a H ion level in the neighborhood of pH 6 to 7.

The large changes in H ion concentration in the alkaline breis are not due to changes in the protein molecule under the influence of the alkali. This we infer from the fact that when egg albumin

solutions are made up to similar pH levels they show no changes on standing comparable to those found in the liver mixtures. The changes then must be due to the production of acids such as CO_2 , lactic acid, fatty acids, and phosphoric acid.

Acids Developed in Tissues Post Mortem.

In a subsequent paper we shall present in more detail studies of the acids produced in tissues made up to various pH levels. In Table VI is shown the relative importance of the various acids in a liver made definitely alkaline with NaOH.

TABLE VI.*
Acids Developed During Autolysis of Pork Livers.

Acid.	0.2 N acid.		Total acid.	
	Initial.	48 hrs.	Initial.	48 hrs.
	cc.	cc.	per cent	per cent
Total fatty acids (by saponification of fats)...	25.9	25.9		
Fatty acids free in brei; <i>i.e.</i> , as soaps.....	3.5	10.7	14.6	15.8
CO_2 , calculated as bicarbonate.....	5	13	20.8	19.2
Lactic acid.....	1.5	3.1	6.3	4.6
Phosphoric acid, calculated as 0.2 M H_3PO_4	14	41	58.3	60.4
Total.....	24	67.8	100	100

* First samples taken immediately after making up breis, or about 6 hours after death of the animals. Second samples taken 48 hours later. Incubated at 37°C .

All breis contained 50 cc. of 0.2 N NaOH per 100 gm. of liver, made up to 500 cc. of brei, and preserved with toluene.

It will be seen that the total titratable acids, excluding combined fatty acids, increase nearly 300 per cent in the course of 48 hours incubation. Lactic acid is insignificant in actual amount, but shows a similar increase. Fatty acids are likewise insignificant in this case, but show a corresponding increase on standing. Carbon dioxide is next in importance, while phosphoric acid is almost double all the rest. Phosphoric acid also shows a 300 per cent increase on standing.

The fat content of the liver varies in the individual from time to time, and between species there is an extremely wide variation. While the liver lipase is not very active in the digestion of the

common fats as compared with the pancreatic lipase, there is the probability that hydrolysis of the fats may play a considerable rôle in determining the reaction in liver cells and so of the degree of autolysis. Free fatty acids compete with the proteins of the cell for the bases present and so tend to raise the H ion level, although themselves insoluble. Table VII is illustrative.

TABLE VII.
Pig Liver and Cottonseed Oil.

No.	Condition.		Days.				
			0	1	3	5	11
I	Control.....	Amino-acid..	0.35	1.70	2.10		2.65
		pH.....	6.56	6.38		6.52	
II	" + 25 cc. oil.....	Amino-acid..	0.35	2.30	4.10		6.15
		pH.....	6.35	4.92		4.83	
III	" + 25 " 0.2 N NaOH.	Amino-acid..	0.35	0.55	0.55		0.75
		pH.....	8.90	7.75		7.37	
IV	" + 25 cc. oil + 0.2 N NaOH.....	Amino-acid..	0.35	0.60	0.95		2.10
		pH.....	8.85	7.54		5.40	
V	Control + 25 cc. rancid oil ..	Amino-acid..	0.35	2.25	3.50		4.70
		pH.....	5.50	5.48		5.43	
VI	" + 25 cc. rancid oil + 0.2 N NaOH.....	Amino-acid..	0.35	1.60	2.35		3.45
		pH.....	6.48	6.26		6.11	

The presence of the fat very definitely increased the H ion level attained by the brei at the end of a day and at later points of time, and increased autolysis quite largely. In the alkaline digests with cottonseed oil, the presence of the fat was not sufficient to increase the H ion concentration to the point where significant digestion could take place. On the other hand, the rancid coconut oil raised the initial H ion very largely and was sufficient to neutralize the effect of the alkali. Digestion in this case was consequently large.

In Table VIII is seen the effect on autolysis of a number of common oils which with one exception definitely increase the extent of autolysis in the time allotted. It is probable that castor oil is not readily hydrolyzed by the liver lipase.

It seems clear that the amount of fat in a liver may have something to do with the individual variations which we have observed in the H ion changes and the autolysis characteristic of individuals. It cannot account for the very rapid initial increase of the H ion, however, following death.

TABLE VIII.
Pig Liver and Various Fats.

No.	Condition.	Days.		
		0	1	8
I	Control.....	0.25	1.70	3.10
II	" + 25 cc. cottonseed oil.....	0.25	1.80	4.20
III	" + 25 " peanut oil.....	0.25	1.90	4.00
IV	" + 25 " cod liver oil.....	0.25	1.95	4.00
V	" + 25 " cocoanut oil.....	0.25	2.10	4.25
VI	" + 25 " castor oil.....	0.25	1.70	3.15
VII	" + 25 " sperm oil.....	0.25	2.10	4.65
VIII	" + 25 " corn oil.....	0.25	1.85	4.40

Quantitative Relation between Acid and Autolysis.

It has been our hypothesis presented in previous papers that the effect of acid was to increase substratum upon which the proteases of the tissue could then act, and that there was little evidence of the phenomenon of activation of the enzyme at some definite H ion level. We showed in 1915 (10) that peptone would digest just as well in the control liver brei as in a brei made acid. Egg albumin which does not digest at all in the control will digest in a brei made acid with HCl, but will not in a brei made acid with MnCl₂. Liver tissue itself, however, digests very well when made acid with MnCl₂. Such facts led us to conclude that the enzyme or enzymes concerned in autolysis were active whether the liver was neutral, alkaline, or acid, but that only in the acid condition was there any substratum present on which the enzymes could demonstrate activity. While the main point is still, we believe,

entirely correct, the recent work of Dernby (7) together with our own offers a more satisfactory explanation of the facts and furnishes some evidence of an activation phenomenon. The ereptic enzyme of the tissue is active in alkaline, neutral, and acid media; the primary protease, however, is only active in acid media. If there are any primary cleavage products present in the tissue, the ereptic enzyme will carry on the cleavage regardless of the reaction, within the limits pH 8 to 3. The cleavage of the tissue proteins themselves, however, takes place only in acid media in which the primary protease is active, and in which also the tissue proteins are converted into acid-protein salts which are available for digestion. Dernby found that gelatin liquefies between the limits pH 6 to 3, and best at pH 4. This is convincing evidence of the presence of this primary protease as distinct from the peptone-digesting action which goes on best at pH 7 to 8. Our own results (8) confirm the interpretation given by Dernby. Peptides containing reactive tyrosine are split off from the tissue proteins very rapidly. They represent the initial break-up of the native proteins into polypeptide molecules of considerable size in which, however, the tyrosine will react with the Folin-Denis reagent. Colorimetric determinations of tyrosine, therefore, make excellent criteria of primary cleavage. It reaches its maximum early in autolysis, long before the amino-acid titer has even approximated equilibrium. At pH 4.5 tyrosine splits off best, at pH 7 and 2.6 it is not split off at all. This can only be explained as due to an enzyme carrying on primary cleavage within that range of H ion concentration.

If a tissue is made acid for 24 hours and then made neutral or alkaline, autolysis proceeds undiminished. During the period of acid reaction the bulk of the tissue is converted into products of primary cleavage which are then available for further cleavage by the erepsin at a H ion level which easily distinguishes it from the primary protease. We are thus led to the conclusion that there must be at least two enzymes present differing in their substrata and in the H ion level at which they are active.

The activity of the enzymes alone is not sufficient to account for the effect of added acid. As has been shown, the production of acid incident to death, and we believe also to injury, is sufficient to give a reaction in which the enzymes are all active, but the

degree of digestion depends on a further factor which is believed to be the *quantity* of acid present, and is without relation to the H ion level attained. In the following experiments typical data are given on which this conclusion rests.

Hydrochloric acid was added to our standard liver or kidney digests in increasing amounts. The digests were allowed to run for periods sufficiently long so that the reaction had practically attained its final point of equilibrium. The amount of amino-acids found at this stage of the reaction measures fairly accurately the amount of digestion possible under the given conditions. The relation, if one exists, between quantity of acid and quantity of protein digested can then be determined. Since the tissue always develops a certain amount of acid itself, the increments of HCl are added to this unknown initial acidity, and this complicates to some extent the interpretation of the results. In spite of this factor, and the recognized limitations of the quantitative determinations of amino-acids by the method used, the results seem fairly conclusive. There appears to be a linear relation between the acid added and the amino-acids finally developed in autolysis. Table IX shows a typical experiment.

Examination of the effect of added acid at different periods, and reduced to the effect of 1 cc., shows how closely alike the effect is through a range of 25 cc. of 0.2 N HCl. Only in the very prolonged digest is there found any falling off in the effect at 25 cc. There is none at 20 cc. This means that in this particular case the addition of acid has produced a quantitative increase of substratum.

In Table X the results of a number of such digests are summarized. Individual differences in livers will account for the differences in results. In some livers 15 cc. produce the maximum digestion. More acid has little or no effect because all the protein which acids can convert into substratum has been converted. The striking feature of this series of digests is the fact that the average figures at 5, 10, and 15 cc. agree so closely with each other.

If acetic acid is added, or any other weak acid, we find an effect in every way similar to that produced by HCl, up to the point where maximum digestion occurs. Beyond this point HCl produces marked inhibition, presumably by destruction of the enzyme. Acetic acid, however, with its relatively low dissociation

does not raise the H ion level to a point destructive to the enzymes even when added in very large amounts. In Fig. 3 the effect of the two acids is compared.

TABLE X.

Amino-Acids Referable to 1 Cc. of Added 0.2 N HCl, at the Intervals of 5, 10, 15, and 20 Cc.

Digest.	Hydrochloric acid.					
	5 cc.	10 cc.	15 cc.	20 cc.	25 cc.	30 cc.
I	0.14	0.14		0.13		0.083
II	0.105	0.106		0.083	0.66	
III	0.07	0.105	0.116	0.103	0.080	0.07
IV	0.18	0.15	0.15	0.15	0.11	
V	0.15	0.17	0.13	0.11	0.086	
VI	0.17	0.18	0.17	0.122		
VII	0.18	0.16	0.14	0.115		
Average.....	0.142	0.147	0.141	0.116	0.083	0.07

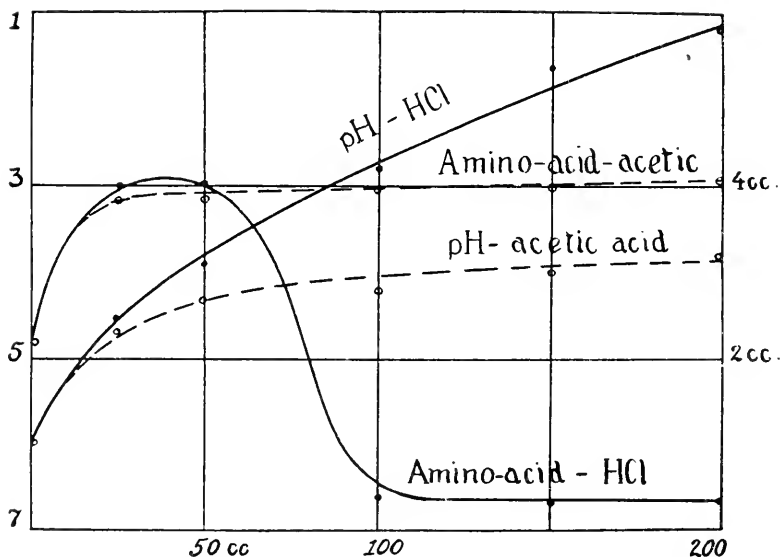


FIG. 3. Effect of HCl and acetic acid on pH of liver, and upon autolysis.

Effects of Various Acids.

In Tables XI and XII we have compared various acids added in equivalent amounts. They represent a wide range of dissociation, but all produce maximum autolysis at the equivalent concentrations.

TABLE XI.

No.	Condition.	Days.				
		0	1	3	8	30
I	Control.....	0.30	1.60	2.20	2.25	2.75
II	" + 35 cc. 0.2 N HCl....	0.30	2.85	4.50	4.80	5.50
III	" + 50 " 0.2 " " ...	0.30	3.30	4.90	5.30	5.30
IV	" + 80 " 0.2 " " ...	0.30	2.20	3.35	4.00	4.20
V	" + 100 " 0.2 " " ...	0.30	0.40	0.55	0.60	0.70
VI	" + 35 " 0.2 " acetic.	0.30	2.60	4.70	4.80	5.90
VII	" + 50 " 0.2 " " "	0.30	2.60	4.85	4.90	5.70
VIII	" + 80 " 0.2 " " "	0.30	2.85	4.70	4.80	5.70
IX	" + 100 " 0.2 " " "	0.30	3.15	4.90	4.75	5.55
X	" + 35 " 0.2 " oxalic.	0.30	3.55	4.80	5.40	5.60
XI	" + 50 " 0.2 " " "	0.30	3.70	4.90	5.20	5.60
XII	" + 80 " 0.2 " " "	0.30	3.35	4.80	5.00	5.60
XIII	" + 100 " 0.2 " " "	0.30	2.75	4.50	4.60	5.40
XIV	" + 35 " 0.2 " buty- ric.....	0.30	2.65	4.40	4.60	5.50
XV	Control + 50 cc. 0.2 N buty- ric.....	0.30	2.50	4.45	4.50	5.90
XVI	Control + 80 cc. 0.2 N buty- ric.....	0.30	2.60	4.50	4.55	5.70
XVII	Control + 100 cc. 0.2 N buty- ric.....	0.30	2.60	3.45	4.60	5.80

The above experiments do not agree with the results reported by Arinkin (2) who believed acids accelerated in some way the enzymic activity, and each to a characteristic degree independent of concentration or equivalence. His own figures, however, show that there was better agreement in the results when equivalent amounts of various acids were added than when similar percentages were present. Arinkin measured autolysis by determining

the N in the filtrates after heat coagulation. When such figures are obtained in the very early stages of autolysis they represent initial cleavage only; later, they represent initial cleavage completed and secondary cleavage under way; after a week or more, they represent total autolysis and correspond quite well with determinations of amino-acids. Arinkin's results, therefore, are not necessarily at variance with our own, though his conclusions are.

The figures presented in Tables IX to XII appear to substantiate the idea that the cell proteins are modified by acids stoichi-

TABLE XII.

No.	Condition.	Days.					Gain due to acid.
		0	1	2	7	32	
I	Control.....	0.30	1.65	1.90	2.50	2.80	
II	" + 35 cc. 0.2 N HCl.....	0.30	2.75	4.05	5.05	6.00	2.90
III	" + 35 " 0.2 " citric.....	0.30	2.95	4.20	5.25	6.20	3.10
IV	" + 35 " 0.2 " tartaric.....	0.30	2.80	3.85	5.10	6.10	3.00
V	" + 35 " 0.2 " formic.....	0.30	2.65	3.75	4.90	6.00	2.90
VI	" + 35 " 0.2 " butyric.....	0.30	2.45	3.55	4.90	6.00	2.90
VII	" + 35 " 0.2 " acetic.....	0.30	2.70	3.70	5.00	6.25	3.15
VIII	" + 35 " 0.2 " lactic.....	0.30	3.10	4.20	5.00	6.15	3.05
IX	" + 0.52 gm. mucic.....	0.30	3.45	3.80	5.00	6.10	3.00
X	" + 0.69 " salicylic.....	0.30	2.50	3.30	4.65	6.00	2.90
XI	" + 0.61 " benzoic.....	0.30	2.40	3.45	4.50	5.95	2.85

ometrically, as shown by Loeb (11) and others (12), and that this modified acid-protein salt or its ions constitute the actual substratum in autolysis.

SUMMARY.

1. Following death the H ion concentration of liver cells increases with almost explosive rapidity. A maximum of pH 6 (average) is reached in 4 to 48 hours, after which the H ion concentration sinks to about pH 6.6 in 10 days. Thereafter, a slow increase of acidity is noticed.

2. If acid is added the initial high H ion concentration sinks slightly for a few days as hydrolysis goes forward and the bulk of the proteins are converted into amino-acids. Thereafter, there is little change.

3. If alkali is added a very rapid increase of the H ion follows. This may be sufficient to change the pH from 9 to 7 + in 24 to 48 hours. The more alkali added the more extensive is the change in H ion.

4. Phosphoric acid accounts for the larger part of the increasing H ion concentrations of control and alkaline breis.

5. Fatty acids are produced in autolyzing liver and by competing with the proteins for the basic groups, contribute to the increased H ion concentration. If common fats are added to a liver they raise the H ion level and increase autolysis.

6. The addition of acids in equivalent amounts causes a proportionate increase in amino-acids produced. The relation between acids added and extent of autolysis appears to be a definitely stoichiometric one. The optimum point in autolysis is reached when acid has been added sufficient to convert all the tissue proteins, susceptible of conversion, into the acid-protein salt. This constitutes the substratum for the autolytic enzymes.

7. Strong acids added in excess of the amount necessary to produce maximum autolysis, inhibit digestion. Weak acids added also in excess produce no inhibition. The enzymes appear to be destroyed at the higher H ion levels produced by the strong acids.

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POSTMORTEM ACIDITY.

I. THE ACIDS FORMED IN AUTOLYZING LIVER.

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The reaction of mammalian tissues is usually supposed to be equivalent to or near that of the blood, in the vicinity of pH 7.4. The reaction of liver breis a few hours after death (1) is from pH 6.7 to 6.3. In the following days this may become as acid as pH 6.0 in some cases. If to such a liver brei sufficient sodium hydroxide is added to make the brei 0.02 N the reaction is near pH 9. In the course of the following 2 days this reaction approaches pH 7.0, rapidly at first. The neutral point is not actually reached after even a month of autolysis. If a similar brei is made 0.02 N with hydrochloric acid the initial reaction is displaced to about pH 4. Acid thus produces about as great a change in reaction as the equivalent amount of alkali. As autolysis proceeds the acidified brei also approaches neutrality, but much less rapidly than the alkaline brei. The limit of this decreasing acidity is about pH 5. Evidently the brei not only contains preformed buffers for the alkali or acid, but it develops still further buffers for both. This paper is concerned with the nature of these buffers developed post mortem.

It is reasonable to suppose that the amino-acids and simple peptides liberated in autolysis are a factor. These compounds would be expected to buffer alkalies and acids since they dissociate both as acids and alkalies. A mixture of amino-acids obtained by hydrolysis of the liver may be expected to have approximately as much buffering action for one as for the other. The pH measurements, however, show that very small changes of reaction occur in the acid breis where large amounts of the amino-acids are being liberated. The increased buffer effect of amino-

acids over the protein itself against acid is thus seen to be small, or counteracted by the production of other acids in the digests. In the alkaline breis where there is no digestion of protein going on the reaction is found to change most markedly. We are therefore led to believe that some stronger acids are liberated in the course of autolysis. Lactic acid is usually supposed to be responsible for the acidity of tissues post mortem. Carbonic acid, phosphoric acid, and the fatty acids are other possibilities to be studied.

Fatty Acids.—The fatty acids would not directly affect the H ion concentration because of insolubility. They will combine with the bases in alkaline digests, however, if hydrolysis of the fats occurs and thus tend to raise the H ion level at least to that of a dilute soap solution. As a matter of fact it has been shown in previous work from this laboratory (1) that fatty acids will raise the H ion of a normal brei through a range of about pH 6.6 to 6.4. The fatty acids liberated in an autolyzing liver are, therefore, theoretically important in proportion to the amounts present. The soaps formed should also tend to buffer a rising acidity due to water-soluble acids formed. To evaluate the fatty acid effect a series of pig liver breis was set up in the usual manner and kept at 36°C. When varying amounts of liver were used the same proportions of water, toluene, and other added materials were employed as in the other autolysis experiments. Breis were neutralized to litmus with phosphoric acid, boiled, and filtered. The fat and fatty acid content were determined by Soxhlet extraction of the dried coagula. Free fatty acids were titrated directly, combined fatty acids by the saponification method. The results in both titrations are reported in terms of cc. of 0.2 N fatty acid per 100 gm. of liver, in Table I.

The amounts of fatty acid liberated in the brei are to be compared with the amount of alkali which such a brei can buffer. Breis made 0.01 N with sodium hydroxide reach approximately pH 7.0 at the end of 2 days; the 0.02 N breis are between pH 7.0 and 8.0. These breis contain 25 and 50 cc. of 0.2 N alkali per 100 gm. of liver. Fatty acids, therefore, may be said to have a small but not inconsiderable part in buffering the alkali. Their importance may be exaggerated here because small amounts of lactic acid or other acids soluble in both water and ether may be

included. On the other hand, the use of phenolphthalein for these titrations tends to reduce the amount of acid found. The use of thymolphthalein to give a true end-point (2) was not possible because of the deep yellow color of the alcoholic solutions. It will be noted that the fatty acids free in the digests increase with time as would be expected from the known lipolytic activity of the liver. The neutral fats, however, do not decrease. This means that the cytolytic and dispersing action of the alkali makes more total fat available with time, just as happens in enzymatic digestion of a tissue (3).

Water-Soluble Acids.—To gain an idea of how much water-soluble acidity there is formed in autolysis a series of breis was set up with pig liver about 6 hours after death of the animals. The

TABLE I.

Brei content.	Time of autolysis.	0.2 N fatty acid per 100 gm. liver.	
		Preformed.	From fats.
	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>
200 gm. liver and 0.01 N NaOH.	0	6.9	17.2
	20	8.6	21.2
	48	11.4	19.4
200 gm. liver and 0.02 N NaOH.	0	3.5	25.9
	20	7.8	25.1
	48	10.7	25.9

breis were boiled to obtain clear filtrates. The breis to which acid or alkali had been added were neutralized by adding the exact equivalent of sodium hydroxide or sulfuric acid before heat was applied. The coagulum was twice boiled in about 400 cc. of water and the filtrates were combined. For titration the mixed filtrate was divided into halves which were titrated with 0.2 N alkali, using phenolphthalein. One-half served as a color blank for the other. Both halves were titrated. In twenty-one such determinations the greatest deviation from the mean of a pair was 3.3 per cent, while the series averages 1.6 per cent deviation. In Table II are shown the results of two such series.

The initial amounts of acid in the two series and for control, acid, and alkaline breis are strikingly similar. The marked

increases are seen in the first 24 hours, including probably some survival processes (4) as well as autolytic changes. The acidified breis develop far more acid than the others, and there is no indication of a maximum in the 48 hour periods studied. In the alkaline and in the control breis 31 to 33 cc. of 0.2 N acid are found after 48 hours. Comparison with the 50 cc. of alkali added to the alkaline breis shows that over 60 per cent of this has been neutralized by water-soluble acids. This titration is significant because the actual H ion concentration in these breis at this time is about pH 8.0, near the end-point for phenolphthalein.

TABLE II.

Brei content.	Time of autolysis.	0.2 N acid.			
		Fixed water-soluble.		CO ₂	Total.
		(1)	(2)	(2)	(2)
	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
100 gm. liver.	0	14.8	13.9	5	18.9
	24	29.9	30.7	8	38.7
	48	30.2	30.7	6	36.7
100 gm. liver and 50 cc. 0.2 N NaOH.	0	15.3	14.5	5	19.5
	24	31.7	30.7	10	40.7
	48	31.0	33.0	13	46.0
100 gm. liver and 50 cc. 0.2 N H ₂ SO ₄ .	0	12.3	14.2	5	19.2
	24	63.9	61.3	4	65.3
	48	91.8	78.8	7	85.8
	120		88.0	8	96.0

Carbonic Acid.—Although it is evident that water-soluble fixed acids are the most considerable factor in the acidity of the tissue and in the buffering of alkali added to a brei, carbon dioxide must be a factor in both. It was boiled off before the titrations were made. Carbon dioxide was determined in these same breis after mixing, but before heat was applied. The digestion bottles were stoppered, though not absolutely gas-tight. The simple and acid digests have therefore lost a small amount of their carbon dioxide, while the alkaline digests have had no increase absorbed from the air. The determinations of carbon dioxide were made by measuring a sample of the brei into a glass

stoppered burette, 2 cc. of 20 per cent tartaric acid were added, the burette was stoppered and rotated for 2 minutes with the axis horizontal. This equilibration burette was connected with a gas burette, the air displaced into the latter by acidulated water, and the carbon dioxide content determined by the decrease in volume after shaking with alkali. Below pH 8.0 carbon dioxide is assumed to be in combination as bicarbonate. From the volume of gas measured, corrected to standard conditions, we have calculated the cc. of 0.2 N CO_2 as BHC'O_3 . These values should then be added to the water-soluble fixed acids to give the totals. In Table II are given the results of such determinations. The last column shows the total of the water-soluble fixed acids and the carbonic acid in the same experiment. This analysis emphasizes the importance of the fixed water-soluble acids.

Lactic and Phosphoric Acids.—The acidified digests produce far more acid than the controls or the alkaline samples. Yet the H ion concentration of the acid breis is known to return somewhat toward the neutral point from the very beginning of autolysis. This change is in the range of pH 4.0 to 5.0. The acids formed must consequently be largely water-soluble weak acids. Lactic and phosphoric acids are to be considered. For the determinations of these acids the breis were freed from protein by the use of mercuric chloride in hydrochloric acid solution (5). Portions of the protein and mercury-free filtrates were reserved for the determination of the inorganic phosphorus content. The larger portions of these filtrates were concentrated on the water bath, keeping the reaction slightly acid. The sirupy concentrate was acidified with 10 cc. of concentrated phosphoric acid, mixed with plaster of Paris, and allowed to dry thoroughly. This mass was powdered and then subjected to continuous hot ether extraction for 16 hours or more (6). The residue after evaporation of the ether was made up to 100 cc. with water and tested for β -hydroxybutyric acid (7). In no case was a trace found. The liquid was used for the determination of the lactic acid by the von Fürth-Charnass method (8). In the first series the preparation of the zinc lactate was omitted; in the second the complete technique was followed. The second series is therefore more accurate, since the disturbing presence of phosphoric acid is eliminated. The method was found to yield 95 and 101 per cent of the theoretical with pure zinc lactate; the author's, averages only 89 per cent.

Inorganic phosphorus was determined by the Bell-Doisy method for blood phosphorus (9), using the protein and mercury-free filtrate instead of the trichloroacetic acid filtrate. The results are calculated to mg. of phosphorus per 100 gm. of liver. In the H ion range covered by these breis phosphoric acid acts as a mixture of a mono-basic and a di-basic acid (10). Not until the reaction is about pH 9.0 does all the phosphoric acid become di-basic. It is purely mono-basic when more acid than pH 4.5. The simplest approximation is, therefore, to calculate the phosphorus

TABLE III.

Brei content.	Time of autolysis.	Acid liberated per 100 gm. liver.							
		Lactic acid.				Phosphoric acid.			
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
	hrs.	mg.	mg.	cc. 0.2 N	cc. 0.2 N	mg. P	mg. P	cc. 0.2 M	cc. 0.2 M
100 gm. liver.	0	115	29	6.4	1.6	94	95	15	15
	24	79	46	4.4	2.6	175	105	28	17
	48	166	46	9.2	2.6	217	212	35	34
	120		67		3.7				
100 gm. liver and 0.02 N H ₂ SO ₄ .	0	117		6.5		96		15	
	24	268		14.9		134		22	
	48	42		2.3		150		24	
100 gm. liver and 0.02 N NaOH.	0		26		1.5		86		14
	24		25		1.4		136		22
	48		56		3.1		254		41
	120		18		1.0		266		43

to cc. of 0.2 M acid, and use these figures for comparison with 0.2 N acid and alkali. This method underestimates the alkali-combining power of phosphoric acid. The real power is between one and two times as great as these figures indicate, depending on the H ion level in any particular case. In Table III are presented some results of such analyses in two series.

As mentioned above, the second series of determinations of lactic acid is more trustworthy, due to the use of the complete technique. It is perhaps surprising to note how little of the acidity of the breis may be attributed to lactic acid. Although

both the control and alkaline breis show increases in their lactic acid content this is almost insignificant as compared with the titrated acidity of similar breis. On the other hand, the phosphoric acid shows as great or greater increases relatively, and when these amounts are calculated to 0.2 M acid the amounts are sufficient to account for most of the titratable acidity.

It is evident that in acid breis the formation of phosphoric acid is definitely retarded as compared with the control breis, while in the alkaline digests the liberation of phosphoric acid is increased. This is remarkable in view of the different conduct of the titratable acidity. The alkaline digests show very little gain in acids over the controls, but the acid digests increase the

TABLE IV.
Relative Importance of Acids in Liver Autolysis.

Acid.	0.2 N acid.		Percentage of total.	
	Initial.	48 hrs.	Initial.	48 hrs.
	cc.	cc.	per cent	per cent
Fatty acids.....	3.5	10.7	14.6	15.8
Carbon dioxide, as bicarbonate ...	5	13	20.8	19.2
Lactic acid.....	1.5	3.1	6.3	4.6
Phosphoric acid, 0.2 M.....	14	41	58.3	60.4
Total.....	24	67.8	100.0	100.0
Water-soluble acids.....	14.9	32.0		

acid content markedly. This increase of acids in the acidified breis must be due to some other acids than carbonic, lactic, phosphoric, or β -hydroxybutyric acids.

The relative importance of the different acids studied may be pointed out by means of Table IV. The figures for the various acids are taken from the preceding tables. To simplify comparison only those breis are considered which were made 0.02 N with alkali. The initial sample and the 48 hour sample are placed in the adjacent columns to show the increases in the buffers for alkali. The sum of the four acids represents the total buffer for the added alkali, assuming that there are no other acids concerned. Using this total as 100 per cent the relative importance of the four acids is shown in the last two columns in per cent.

It is reasonable to assume that there are no other acids of importance in buffering the 50 cc. of 0.2 N sodium hydroxide added. If 50 cc. of 0.2 N strong acid were added to such an alkaline brei its H ion should be near that of the control brei. The alkaline breis never become actually acid, even though the amounts of various buffers may more than equal the molecular equivalents of alkali added. The reason is obviously that the buffering acids are weak, and the salts are hydrolyzed with the maintenance of an alkaline reaction. As the reaction becomes more acid than pH 8.0 the buffering due to fatty acids and carbon dioxide decreases in importance and that due to phosphoric acid and lactic acid becomes relatively more important. After 48 hours the sum of the lactic and phosphoric acids is more than enough to account for the titratable water-soluble acid in similar digests. The lack of more exact agreement is not surprising in view of the method of determining phosphoric acid by calculation from the content of inorganic phosphorus, and of the use of separate liver samples for the different determinations.

It is apparent that all four of the acids studied increase in liver autolysis. The order of importance of the four acids does not change in the 48 hour period. But lactic acid becomes less important than at first, while phosphoric acid and the fatty acids become more important. Most striking is the predominant importance of phosphoric acid throughout. The lactic acid is probably the result of survival processes (4), but the liberation of fatty acids and phosphoric acid are processes that continue with autolysis.

Most of the previous work on lactic acid formed in autolyzing tissues is subject to Fletcher's criticisms (4) of infection of the digests or to the confusion of survival processes with a continuing autolysis. Ssobolew (11) found that in the autolysis of liver lactic acid increases from 31 to 88 cc. of 0.2 N acid per 100 gm. of liver. His method, however, included all water-soluble acids, and, consequently, phosphoric acid. The amounts of lactic acid found in the experiments described in this paper agree well with results obtained from dog and rabbit livers. Using the older zinc lactate method with care, dog livers perfused with blood were found to contain 55 to 98 mg. or 3 to 5.5 cc. of 0.2 N lactic acid per 100 gm. of liver (12). More recently, using the complete

method of Embden (5), rabbit livers were found to contain an average of 36 mg. or 2 cc. of 0.2*N* lactic acid per 100 gm. of liver (13). The amounts of phosphorus liberated in autolyzing liver have been confirmed in work appearing since these experiments were completed (14). Using standard gravimetric methods the inorganic phosphorus content of pig liver after 4 days autolysis was 191 mg. per 100 gm., after 8 days it was 260 mg. per 100 gm. of liver. These values are of the same order of magnitude as those given for the 48 and 120 hour determinations in Table III.

SUMMARY.

The acid content of sterile autolyzing pig liver increases with time. In simple breis of water and liver, and in those made alkaline this increase is largely complete within the first 24 hours of autolysis. In acidified breis the increase goes on for a considerably longer time, and with the production of a larger amount of acid. The amounts of acid produced in the simple and alkaline breis are similar. In alkaline breis the relative importance of the acids is: phosphoric, carbonic, fatty, and lactic acids. The phosphoric acid represents more than the others combined. In the water-soluble and non-volatile acidity of liver, phosphoric acid is at least ten times as important as lactic acid.

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POSTMORTEM ACIDITY.

II. PHOSPHORIC ACID LIBERATION IN LIVER AUTOLYSIS.

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Since phosphoric acid is the most important fraction of the acidity of liver post mortem (1), it is of interest to study in detail the factors which control the development of this acid. The standard liver digests employed in the earlier work were also used for this study. Digestion was stopped and the proteins were precipitated by the use of trichloroacetic acid. The inorganic phosphorus content of the filtrates was determined by the Briggs modification of the Bell-Doisy method (2). The method is far more satisfactory than the original Bell-Doisy method, since there is no fading. The range over which the color intensity is accurately proportional to the amount of phosphorus present is from 0.5 to 2.0 times the amount used as a standard. It is desirable that the time allowed for color development should be an hour rather than a half hour as stated by Briggs. The full color is developed by the end of an hour; it is 94 per cent complete in half an hour. There is no further intensification of the color by the end of 3 hours. Heat cannot be used to accelerate this reaction. If the mixed reagents are heated in boiling water for even 2 minutes, the molybdic acid is reduced with the production of an intense blue color. This reduction of molybdic acid by hydroquinone takes place at room temperatures, but so slowly that a blue color can be detected only after 4 hours, using the reagents in the proportions for the phosphorus determination.

Dog livers were used for the experiments here reported, in order to secure material for study from the very first moments after death. Preliminary studies showed that the increase in the

inorganic phosphorus content was most rapid at this time. A dog was shot through the brain, bled at once from the neck, and the liver removed, ground, and a series of breis prepared. One was used as a control, one was made 0.02 N with sodium hydroxide, another 0.01 N with sulfuric acid, and another 0.02 N with acetic acid. Initial samples were taken from each as rapidly as possible, and at intervals afterward. The development of the inorganic phosphorus is shown in Table I and Fig. 1, typical of several such experiments.

The curve for the brei made acid with sulfuric acid is not shown because it is almost identical with that for acetic acid. The three upper curves represent the entire course of the autolysis for 20 days. The lower curves present the same data for the first 6½ hours only, plotted with the same scale of phosphorus on the

TABLE I.
Inorganic Phosphorus in Mg. per 100 Gm. of Liver.

Time after death.....	Hrs.					Days.		
		1	2	6½	24	2	6	20
Control.....	21.4 (10 min.)	59.7	80.6	119	178	201	272	292
0.02 N acetic acid.....	22.1 (10 min.)	64.5	77.5	96	148	183	237	259
0.01 N H ₂ SO ₄	55.5 (25 min.)	62.0	77.0	94	141	190	239	266
0.02 N NaOH.....	37.4 (20 min.)	61.5	83.3	138	211	250	298	310

ordinate axis, but with the time scale magnified 48 times. The curves show a distinct increase in the amount of phosphorus liberated in the alkaline breis, and a corresponding decrease in the amount formed in acid breis. This variation from the control curves becomes definite after the 1st hour. The differences are maintained indefinitely. These differences between acid, alkaline, and control digests are largely complete by the end of the 1st day of digestion. This may be explained as a survival process, analogous to the formation of lactic acid in surviving muscle (3). In the latter case the presence of alkali makes possible the production of more acid, the presence of acid depresses the liberation of lactic acid.

If the curves in Fig. 1 are projected backward toward the time of death they would indicate that the content of inorganic phosphorus in the tissue in life must be less than 10 mg. per 100 gm.,

or of the order of magnitude of that in the blood. Attempts were made to decrease still further the time between death and the initial sampling of the brei. It seems possible that when death is caused by a shot, or any other similar trauma, the violent nervous stimulation that always ensues might well cause an explosive metabolism of phosphorus in the liver just as it causes violent muscular contraction with the liberation of lactic and phosphoric acids. To minimize this effect a dog was etherized, the liver

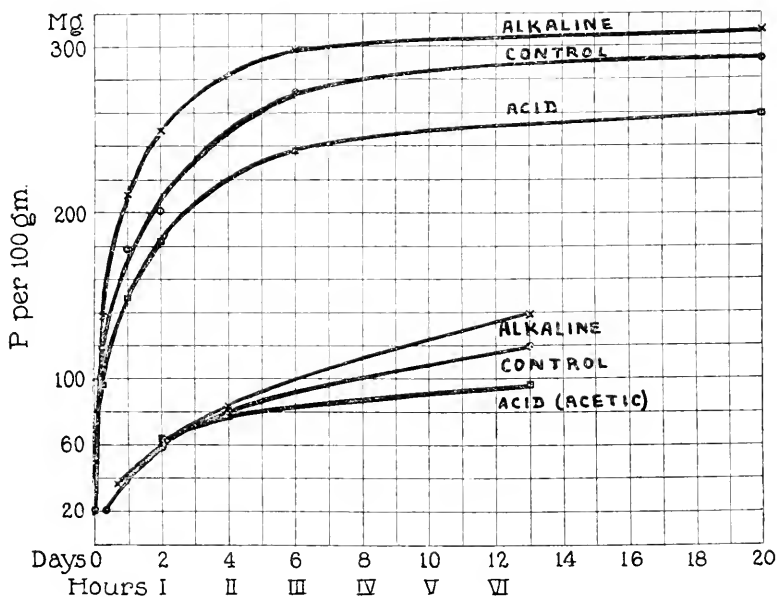


FIG. 1.

ligated with minimal handling, and excised while the animal was alive. The curve and the data from a typical experiment are given in Table II and Fig. 2.

In such experiments, with more frequent sampling of the breis than before, it became apparent that the curves cannot be legitimately projected backward. There is an initial period of about half an hour in which the inorganic phosphorus content of the brei shows little or no change. The same result was obtained in a dog killed an hour and a half after the spinal cord had been cut in the

upper dorsal region. These determinations do, however, give the lowest initial inorganic phosphorus contents of dog liver, 12.6, 15.0, and 19.7 mg. per 100 gm. This initial lag in the curve need not be interpreted in terms of nervous excitation or enzyme activation. It is a temperature effect.

This production of inorganic phosphorus is enzymatic, for it may be arrested by boiling the brei. If a liver brei is prepared and

TABLE II.

Time after removal of liver.....	Min.					Hrs.			Days.	
	6	10	17	32	51	2½	6	24	7	125
Inorganic phosphorus.....	12.6	12.7	12.6	25	37.4	71	116	149	207	261

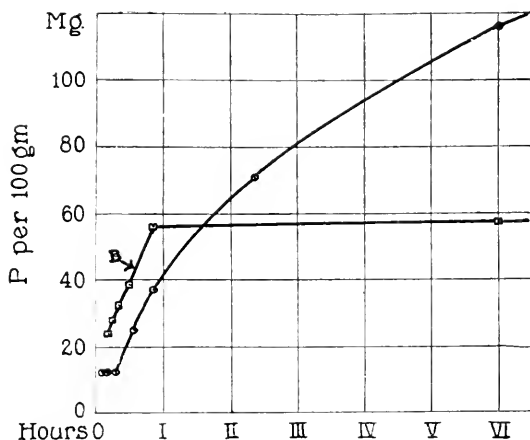


FIG. 2.

sampled several times during the first half hour, then boiled and the sampling continued, the increase is no longer apparent. In Table III are data from such an experiment. The results appear graphically in Fig. 2. At the point marked "B" the digest was heated to boiling. The increased phosphorus content after 6 and 120 days incubation is no more than may be expected from the spontaneous hydrolysis of such an acid brei kept at 36°C.

Further analysis of the effect of temperature was necessary. The work so far had been done at usual laboratory temperatures, with all utensils and reagents at about 20°C. The lag in the increase of inorganic phosphorus is really due to the chilling of the tissue by postmortem handling, followed by an increase as the digest is warmed up in the incubator. This was demonstrated as follows: A dog was shot, bled, and carried at once into a room maintained at 5°C. All the utensils and reagents for the experiment had been kept in the room long enough to attain the low temperature. The liver was quickly removed and packed in melting snow for 10 minutes. The brei was then prepared as usual but kept at 5°C. After several samples had been taken in the course of the hour following death, the brei was heated to 36°C. in a water bath, and kept as usual at this temperature. Another dog was shot and bled, and the liver removed for the

TABLE III.

	Min.					Hrs.		Days.	
	11	16	20	30	50	6	24	6	12 ¹
Time after removal of liver.....									
Inorganic phosphorus.....	24.1	28.3	32.8	38.8*	56.7	57.5	59.3	65	83.3

* Brei was boiled after 30 minutes autolysis.

usual brei preparation, this time performing the entire experiment in the constant temperature room at 36°C. The results of such a paired experiment are given in Table IV and Fig. 3. The entire experiment was repeated with similar results.

It is apparent that the lag in the curve of phosphorus liberation may be prolonged by lowering the temperature to the point of preventing or minimizing enzyme action.

The concentration of inorganic phosphorus at the moment of death cannot be deduced with certainty from these experiments. There is as much in the liver chilled before being ground as in that ground in the room at body temperature. It would seem reasonable to project backward the curves for phosphorus liberation at body temperature except for the uncertain effect which grinding may have on the process. The lowest concentrations determined were in those animals from which the livers were excised before death, but under anesthesia. In these cases the

digestion was stopped with trichloroacetic acid in 6 to 7 minutes after the removal of the liver from the animal. The inorganic phosphorus content of live and resting liver must be less than these values; *i.e.*, 12.6, 15, and 19.7 mg. per 100 gm. of liver.

TABLE IV.
Inorganic Phosphorus in Mg. per 100 Gm. of Liver.

Time after death.....	Min.							Hrs.			
	9	15	25	35	41	45	60	1½	2	6	6½
°C.											
5			25		24.5		25	24.2*	49		102
36	24.5	30.3	47.6	54		59.2	64.1		80.6	123	

* The 5°C. brei was warmed to 36°C. just before the 1½ hour sample was taken.

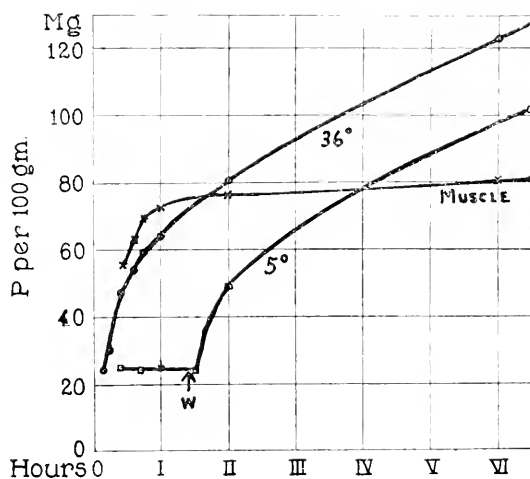


FIG. 3.

In muscle the liberation of inorganic phosphorus follows a different course. In Fig. 3 is shown the curve of the phosphorus liberation in leg muscle of the same dog from which the liver was obtained for the experiment at body temperature. The muscle was handled like the liver in this case. The data for this experiment appear in Table V.

In muscle there is a more rapid rise of the inorganic phosphorus content and an earlier approach to an equilibrium condition than is the case in liver. The muscle breis continue to increase in phosphoric acid as do livers, but at a much slower rate, and with a much lower total phosphorus liberation. The prominent factor here is the early formation of large amounts of phosphoric acid, to be identified with the breakdown of lactacidogen. This process is evidently complete by the end of 2 hours after the death of the animal, as assumed by Embden and coworkers (4).

TABLE V.

Inorganic Phosphorus in Mg. per 100 Gm. of Muscle.

	Min.				Hrs.			Days.	
	28	37	45	60	2	6	21	8	46
Time after death.....									
Inorganic phosphorus.....	56.2	62.9	69.7	72.5	76.9	80.9	104	125	154

SUMMARY.

Dog liver breis prepared and kept at body temperature show a rapidly increasing content of inorganic phosphorus. If the tissue is chilled in the preparation of the brei this increase is delayed. The addition of alkali causes a greater increase, while acid causes less increase than in breis made of liver and water, preserved with toluene. The liberation of inorganic phosphorus is enzymatic, and is thought to include a survival process as well as a more prolonged autolytic liberation of phosphoric acid. The lowest inorganic phosphorus contents recorded range from 12.6 to 19.7 mg. per 100 gm. of liver.

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THE ANALYSIS OF TUBERCULINIC ACID.*

BY ELMER E. BROWN AND TREAT B. JOHNSON.

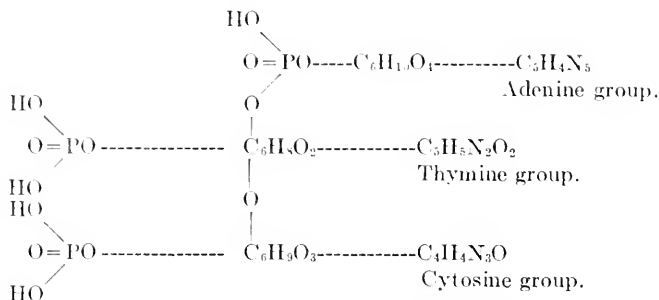
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In a previous publication from this laboratory it has been shown to be possible to isolate from the nucleus of the tubercle bacillus a nucleic acid in amounts corresponding to about 1.5 per cent of the weight of the original bacilli. In this publication we reported no figures indicating the composition of this acid and from the facts which were available at that time we were unable to state whether the acid isolated by us was a true tetranucleotide or a degradation product of a true nucleic acid. In order to obtain further light upon this question we have repeated our work and isolated by our method tuberculinic acid in larger quantities, subjected it to the recommended methods of purification, and submitted our final product to a complete analysis. We have also found that it is not necessary to use alkali for the extraction of the greater portion of the nucleic acid contained in the tubercle bacilli, but that water is a better solvent for this purpose. Aside from the fact that more nucleic acid is obtained when water is used as the solvent, then followed with alkali, a crude product is obtained easier of purification and more representative of the true nucleic acid contained in tubercle bacilli. From the results of our analytical determinations we are now led to the conclusion that the final product from our purification is not a tetranucleotide, but a trinucleotide containing the purine, adenine, and the two pyrimidines, thymine and cytosine, which provisionally may be represented as on the following page.¹

* This work was done under a grant from the Committee on Medical Research of the National Tuberculosis Association.

¹ The manner of linkage of the above nucleotides and the order of arrangement are arbitrarily assigned.



In other words, in the case of tuberculinic acid we are dealing with a substance of marked instability and in the process of purification the guanine nucleus is split off from the molecule by hydrolysis and a stable trinucleotide is the final product of reaction. We have obtained analytical values which are in accord with the carbon, hydrogen, nitrogen, and phosphorus ratios for a trinucleotide complex. Furthermore, we have been successful in identifying and isolating guanine in the filtrate resulting from the purification of our original nucleic acid. It is entirely probable that the original tuberculinic acid, as obtained by us, does not contain all the cyclic bases in the proper proportion for a true tetranucleotide. The fundamental fact is that the guanine nucleus is more loosely bound than the other three and is very susceptible to hydrolytic influences. In the course of our work we have also been able to confirm our original conclusions regarding the presence of thymine and cytosine in this nucleic acid. We have used both hydrochloric and sulfuric acids as hydrolytic agents, but in no case have we been able to detect a trace of uracil as a product of decomposition.

EXPERIMENTAL PART.

The material used in this research was furnished gratuitously by Parke, Davis and Co. and the Mulford Co.,² and represented about 2 kilos of desiccated bacilli. The two samples were worked separately, as described below, in order to obtain data that might serve as a comparison of the behavior of products from different

² The writers desire to express at this time their appreciation of the generosity of these industrial concerns in supplying them with research material.

sources when subjected to identical procedure, and to determine whether a nucleic acid could be obtained from the different products with a definite composition.

When 810 gm. of the Mulford Co. bacilli were defatted with toluene according to the directions given in our previous paper³ 520 gm. of defatted tubercle bacilli were obtained. This corresponds to a loss in weight of 290 gm. soluble in toluene or equivalent to 35.8 per cent of the weight of bacilli used. 900 gm. of the Parke, Davis and Co. product under similar conditions gave 595 gm. of defatted material, and 304 gm. soluble in toluene corresponding to 33.8 per cent of the weight of bacilli used. These percentages of toluene-soluble material are of the same order, but somewhat smaller than the percentages obtained in similar experiments reported in our previous paper. They show that the composition of the bacilli grown by the two concerns in these two cases was quite uniform. The variations can undoubtedly be explained by the conditions used in the growth of the organisms.

The 520 gm. of the Mulford Co.'s defatted tubercle bacilli, obtained above, when treated according to the writers' method³ for the separation of the nucleic acid, using successively 3 and 5 per cent sodium hydroxide solution for extraction, gave 8.4 gm. of crude nucleic acid or a yield of 1.63 per cent of the weight of original tubercle bacilli used. This result is almost identical with that obtained in a similar experiment on a Mulford Co. product and published in our previous paper, where a yield of 0.95 per cent was reported.

The 596 gm. of defatted tubercle bacilli prepared from the Parke, Davis and Co. bacilli were thoroughly triturated in a large mortar with 1,800 cc. of water and the mixture was allowed to stand over night. The solution was then made distinctly acid to litmus with acetic acid to precipitate protein, filtered, and the protein residue finally washed with 400 cc. of water containing 1 per cent of acetic acid. To the acid filtrate, equivalent to a volume of 1,700 cc., 25 gm. of sodium acetate were added, then hydrochloric acid was added until a slight permanent precipitate was produced, and finally an equal volume of absolute alcohol. Care was taken to keep the solution cold during this operation. A precipitate separated immediately, and was separated from the filtrate by decantation and finally filtered, then washed successively with 95 per cent alcohol, absolute alcohol, and dried with ether. 8.7 gm. of an almost colorless nucleic acid were obtained. This product was saved as described later on.

The residue from the water extraction above was treated with 3 and 5 per cent sodium hydroxide solution and the nucleic acid extracted according to the general procedure. We obtained 6.8 gm. of a dark colored product which was apparently nucleic acid. A total yield of 15.8 gm. of nucleic acid was thus obtained, corresponding to 1.75 per cent of the weight of the

³ Johnson, T. B., and Brown, E. B., *J. Biol. Chem.*, 1922, liv, 721.

original tubercle bacilli used. The highest yield previously obtained, using only 3 and 5 per cent sodium hydroxide solution, was 1.5 per cent of the weight of the original tubercle bacilli. The results of this experiment show that over 55 per cent of the nucleic acid, obtained from the above material, was extracted with water. It is possible that even more of the nucleic acid would have been extracted by water had the process been repeated more thoroughly, since the residues usually occlude an amount of solution equal to the weight of the material. In fact, in this particular experiment 500 cc. less of solution were obtained than there was water added.

The fact that more nucleic acid is obtained by first extracting with water followed by treatment with caustic solution, than when caustic solution alone is used, indicates that this nucleic acid is destroyed in part by the alkali treatment. This observation is fully confirmed by interesting data which will be reported later in this paper.

Purification and Analysis of Tuberculinic Acid.

2.2 gm. of the crude nucleic acid obtained from the Mulford Co. tubercle bacilli above were further purified by dissolving in 1 per cent sodium hydroxide solution, neutralizing with acetic acid, and then adding picric acid solution as long as a precipitate was formed. After filtering, the nucleic acid was precipitated from the clear solution with absolute alcohol which had been acidified with hydrochloric acid. The precipitate settled rapidly, and, after decantation of the mother liquor, was transferred to a filter and washed successively with 50 per cent alcohol, 95 per cent alcohol, absolute alcohol, and finally with ether. Since the product thus obtained was colored slightly yellow, it was dissolved again in dilute sodium hydroxide, the solution poured into six times its volume of absolute alcohol to precipitate the sodium salt and the latter filtered off and washed with absolute alcohol. The salt which was in the form of a yellow granular powder was dissolved in water, and the solution acidified with acetic and hydrochloric acids. On addition of 10 per cent copper chloride solution the copper salt of nucleic acid quickly separated and was finally converted into the free acid according to the directions of Levene,⁴ using 5 per cent hydrochloric acid. The resulting acid was washed with alcohol and dried with ether as described above. The nucleic acid was then obtained in the form of a light grey powder and after drying for 2 hours at 110° gave the following results on analysis:

Found. N (Kjeldahl) 11.53.

The phosphorus was determined according to Hausmann's method, by decomposing the sample with sulfuric and nitric

⁴ Levene, P. A., *J. Biol. Chem.*, 1921, xlviii, 177.

acids and then proceeding according to the standard method; namely, precipitating as the phosphomolybdate and converting the latter into magnesium pyrophosphate. 0.1650 gm. of acid gave 0.0478 gm. of $\text{Mg}_2\text{P}_2\text{O}_7$.

Found. P 8.07.

Carbon and hydrogen were determined by mixing the sample with a large amount of copper oxide, using a Thiele mixing tube and the combustion was carried out in the same way as for nitrogen compounds, using a reduced copper spiral at the end of the tube. No difficulty was experienced in obtaining a complete combustion. 0.2495 gm. of acid gave 0.0997 gm. of water and 0.3226 gm. of carbon dioxide.

Found. C 35.26, H 4.44.

Sodium was determined by burning the sample in a weighed silica crucible and converting the residue into sodium *m*-phosphate by means of the blast lamp. After weighing, the residue was dissolved by long boiling with hydrochloric acid and the phosphorus determined in the usual manner and calculated as PO_3 . 0.2028 gm. of the sample, when treated as above, gave an ash weighing 0.0326 gm. (or 16.07 per cent) and 0.0232 gm. of PO_3 (or 11.44 per cent):

Found. Na 4.63.

Nucleic acid.	Analysis.	Atomic ratios.	
		I	II
	<i>per cent</i>		
C	35.26	33.88	45.10
H	4.44	50.80	67.80
N	11.53	9.46	12.67
P	8.07	3	4
Na	4.63	2.32	3.09
O	34.52	24.90	33.20

$\text{C}_{34}\text{H}_{51}\text{N}_{10}\text{P}_3\text{O}_{25}$ for 3 phosphorus; $\text{C}_{45}\text{H}_{65}\text{N}_{13}\text{P}_4\text{O}_{33}$ for 4 phosphorus.

The nucleic acid obtained from the aqueous extract was dissolved in 1 per cent sodium hydroxide solution and acetic acid added until litmus indicated an acid reaction. The protein which separated was removed with a centrifuge, and the nucleic acid finally precipitated, as previously described, with alcohol and hydrochloric acid. This was washed with alcohol and

ether, as above, using the centrifuge to remove the solvent, instead of filtering to prevent contamination of the precipitate with filter paper. When filtered through a funnel and the nucleic acid dried on the filter paper small particles of paper are always occluded in the product. A white powder was obtained containing 11.63 per cent of nitrogen and 6.22 per cent of phosphorus. The material at this stage of the purification gave a negative biuret test, showing that it was free from protein. The phosphorus-nitrogen ratio was therefore 4 to 16.3 or a value agreeing very closely with the result obtained by Osborne and Harris⁵ from the purer preparations of triticonucleic acid. However, the triticonucleic acid preparations always contained much higher percentages of both nitrogen and phosphorus than were obtained by us.

This material was subjected to a further purification process by converting, successively, into the sodium salt, the copper salt, then into the free acid, as previously described, using the centrifuge to separate the precipitate from the solution to avoid filtration throughout the procedure. The light grey powder was dried at 100° C. for 2 hours and a complete analysis made on the product. The ammonia produced from 0.2841 gm. of sample neutralized 25.10 cc. of 0.1 N hydrochloric acid, which is equivalent to a nitrogen content of 12.36 per cent. The phosphorus was determined upon the same sample by acidifying the solution remaining from the Kjeldahl determination with nitric acid, boiling, and then proceeding according to the standard method. This procedure gives identical results with Hausmann's method or the caustic fusion method of treating the sample. 0.0842 gm. of $Mg_2P_2O_7$ was obtained, corresponding to a phosphorus content of 8.24 per cent.

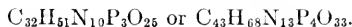
Carbon and hydrogen determination. 0.2412 gm. sample: 0.0985 gm. water and 0.3051 gm. carbon dioxide.

Found. C 34.47, H 4.53.

0.2306 gm. gave an ash weighing 0.0266 gm. (or 11.54 per cent) which was equivalent to 6.69 per cent PO_3 .

Found. Na 4.85.

Nucleic acid.	Analysis.	Atomic ratios.	
	<i>per cent</i>		
C	34.47	32.40	43.20
H	4.53	50.64	67.52
N	12.36	9.96	13.30
P	8.24	3	4
Na	4.85	2.37	3.16
O	35.55	25.06	33.41



⁵ Osborne, T. B., and Harris, I. F., *Z. physiol. Chem.*, 1902, xxxvi, 85.

The above formulas are practically identical with those calculated from the data obtained in the preceding series of analysis, except in percentage of carbon, which was high in the preceding experiment on account of a slight contamination with adsorbed filter paper. The fact that the same product was obtained from both preparations is evidence that it is a definite compound. Furthermore, the fact that analyses show a phosphorus and nitrogen ratio of 4 to 13 or 3 to 10, indicates that the latter ratio is probably the correct one, and that the substance is a trinucleotide, corresponding to the first formula above, calculated on the basis of three phosphorus atoms in the molecule. That this conclusion is correct and that the above product is not the original nucleic acid contained in tubercle bacilli is shown by a consideration of the decomposition products obtained from this material and from the solution from which it was purified.

Decomposition Products of Tuberculinic Acid.

The Purines.

Levene⁶ in an early investigation of the crude nucleic acid from tubercle bacilli was able to isolate from the defatted material precipitates which varied in phosphorus and nitrogen content between 7.79 and 13.09 per cent in the former, and 14.09 to 9.42 per cent in the latter. From these he reported the definite isolation of both guanine and adenine. Long⁷ has more recently investigated the purines contained in defatted bacilli and also isolated both of these purine combinations. The writers, in the light of these investigations, studied first the pyrimidine and sugar content. The pyrimidines were found to be the same and in practically the same proportion as those obtained by analysis of thymus nucleic acid. The sugar was also found to be a hexose,⁸ but the amount we obtained was roughly about three-fourths of the quantity obtained by Steudel⁹ from thymus nucleic acid. The result of our analysis shows that the nitrogen content was too small to account for all the pyrimidines and both purines. On the other hand, since the amount of nitrogen revealed by

⁶ Levene, P. A., *J. Med. Research*, 1901, vi, 135.

⁷ Long, E. R., *Am. Rev. Tuberc.*, 1920-21, iv, 842.

⁸ Brown, E. B., and Johnson, T. B., *J. Am. Chem. Soc.*, 1923, xlv, 1823.

⁹ Steudel, H., *Z. physiol. Chem.*, 1908, lv, 407; lvi, 212.

analysis was sufficient to account for only one purine in the molecule, it became necessary therefore for us to devote attention to this phase of the work in order to see which purine our trinucleotide actually contained.

1 gm. of nucleic acid containing 10.64 per cent of nitrogen and 8.03 per cent of phosphorus was placed in a flask with 25 cc. of 7 per cent sulfuric acid and the mixture boiled for 4 hours under a return condenser by heating in an oil bath at 100–110°C. The filtrate from this digestion was neutralized at the boiling point, with concentrated ammonia and then allowed to stand over night after the addition of small excess (2 per cent) of ammonia. A floccy precipitate settled out, weighing 0.0102 gm. This was filtered and dissolved from the Gooch crucible with dilute sodium hydroxide, the solution made neutral to Congo red with hydrochloric acid, and pieric acid finally added, when a crystalline salt was obtained which melted at 275–280°, and agreed in all its properties with adenine pierate. Hence the product contained no guanine.

To the ammoniacal filtrate obtained above an excess of an ammoniacal solution of silver oxide was added. A voluminous slightly brown colored precipitate was obtained. After standing for an hour it was filtered on an asbestos mat in a Gooch crucible, washed free of ammonia with cold water, transferred to a Kjeldahl flask, and a nitrogen determination made. 0.0297 gm. of nitrogen, corresponding to 28 per cent of the total nitrogen, was obtained. Including the 0.0102 gm. obtained in the guanine fraction as adenine we therefore have 33.21 per cent of the total nitrogen accounted for as adenine. This result proved to us that the missing purine from our purified product was undoubtedly guanine. While the adenine obtained above was not equal to 50 per cent of the total nitrogen as would be required for a trinucleotide composed of thymine, cytosine, and one purine as the nitrogen-containing constituents, yet the sample used for this determination contained a nitrogen content of almost 1 per cent less than that contained in the samples analyzed after careful purification, and this probably accounts for the difference. The above sample of nucleic acid was obtained from the solution after the main fraction of nucleic acid had been separated, and probably had lost some of its adenine.

In order to ascertain if guanine was present in the original material extracted from tubercle bacilli and that it had been removed from tuberculinic acid in the course of purification, the solutions remaining from the precipitations of the material obtained from the Parke, Davis and Co. defatted tubercle bacilli were subjected to further investigation. It was observed that when a portion of the acid was neutralized in the solution from the decomposition of the copper salt a voluminous precipitate was

obtained, and that this precipitate was soluble in 50 per cent alcohol containing 2.5 per cent hydrochloric acid. Since this strength of solution was used in the purification process it was evident that a considerable portion of material could be obtained in this way from the remaining solutions used in the purification process.

To the combined solutions an excess of 10 per cent copper chloride solution was added, then 20 per cent sodium hydroxide solution until a precipitate began to form and the yellow solution changed to the bluish green color characteristic of copper solutions. The resulting fluid was distinctly acid to litmus and a voluminous precipitate was obtained which settled upon standing. After decanting most of the clear liquid, the remainder was removed with the centrifuge. A gelatinous residue weighing several grams was obtained. This product was transferred to a flask, 200 cc. of 5 per cent sulfuric acid were added and heated on an oil bath at 100–110°C. for 5 hours under a return condenser. Since only a small amount of insoluble material separated on cooling, the solution was not filtered but was saturated with hydrogen sulfide, then filtered from the CuS , and the excess of hydrogen sulfide expelled by boiling. To the hot solution, concentrated ammonia was added until neutral. An excess of 4 per cent of ammonia was then added and the solution allowed to stand over night when a colorless distinctly granular precipitate was obtained in appreciable amount. This product was identified as guanine, giving its characteristic picrate which decomposed at 190°C. The quantity obtained weighed 0.1 gm.

To the ammoniacal filtrate from the guanine precipitate an excess of a solution of ammoniacal silver oxide was added and the voluminous precipitate of the silver salt filtered off and decomposed with hydrochloric acid. To the clear filtrate from the silver chloride precipitate, sodium hydroxide solution was added until neutral to Congo red, then an excess of picric acid solution when a precipitate weighing 0.010 gm. was obtained showing that adenine was also present but in very small amount in this solution.

The fact that both guanine and adenine were obtained, the former in a much larger yield, from the purification solutions in amounts as large as the above show that considerable cleavage took place in the purification of the crude nucleic acid, and that no considerable amount of the nucleic acid remained in the mother liquor. This solution contained some of the unchanged acid and the above cleavage products, the small amount of adenine probably coming from the acid, as well as the remaining decomposition products of nucleic acid, phosphoric acid, sugar, and the pyrimidines.

The Pyrimidines.

In the study of the pyrimidines contained in tuberculinic acid several hydrolysis experiments have been carried out using 33 per cent sulfuric acid as the hydrolytic agent. The results of this work have been published in preceding papers and show that in each instance thymine and cytosine were the only pyrimidines found in the hydrolysis solution. In one experiment where the purified nucleic acid was used these two pyrimidines were obtained in yields of 11.8 and 9.7 per cent, respectively. It was also found that only the above pyrimidines were produced, uracil being absent from the hydrolysis products. In an experiment in which concentrated hydrochloric acid was used for hydrolysis instead of 33 per cent sulfuric acid both thymine and cytosine were obtained, while uracil was found to be absent from the hydrolysis solution, as was the case when sulfuric acid was used. These results prove to our satisfaction that uracil is not a cleavage product of tuberculinic acid and is not produced from cytosine under the conditions which we used for hydrolysis.

SUMMARY.

1. Tuberculinic acid has been purified and subjected to a complete analysis for carbon, hydrogen, nitrogen, phosphorus, and inorganic material.
2. The results obtained indicate that the construction of this acid is in conformity with that of a trinucleotide containing adenine, thymine, and cytosine, each of which was obtained from the purified product.
3. Guanine is also a constituent of tuberculinic acid, but is lost from the original molecule in the process of purification.
4. We have been able to confirm our original observation that thymine and cytosine are the only pyrimidine constituents of this nucleic acid.

A MICRO COLORIMETRIC METHOD OF ESTIMATING THE HYDROGEN ION CONCENTRATION OF THE BLOOD.*

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Until very recently the colorimetric method of measuring the hydrogen ion concentration has not possessed the delicacy of other colorimetric methods for the reason that an instrument adapted to the measurement of a combination of two colors, instead of a single color, has not been available. As ordinarily carried out in the past it has been necessary to employ a series of tubes with graduated pH values as standards in the colorimetric determination of the hydrogen ion concentration. The instrument recently described by one of us¹ makes possible as accurate color measurements with a combination of two colors as can be obtained with a standard colorimeter employing a single color. This delicacy is very important in the case of the colorimetric determination of the hydrogen ion concentration of the blood, owing to the fact that the pathological variations are very small. With the method described below the error in color comparison falls within \pm pH 0.02.

The method is essentially an adaptation of the colorimetric technique of Cullen² for the determination of the pH of the blood plasma (or serum) to the bicolorimeter previously described. As

* Presented before the Society for Experimental Biology and Medicine, April 18, 1923 (Myers, V. C., Schmitz, H. W., and Booher, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 362).

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¹ Myers, V. C., *J. Biol. Chem.*, 1922, liv, 675.

² Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

modified, the final determination is carried out on 0.1 cc. of plasma, and does not require more than 10 minutes after the blood has been obtained. By a simple technique contact with the air is entirely excluded.

Method of Taking Blood and Separating Plasma.

Blood is drawn without stasis into a narrow 5 cc. Luer glass syringe containing sufficient mineral oil to fill any air spaces, and

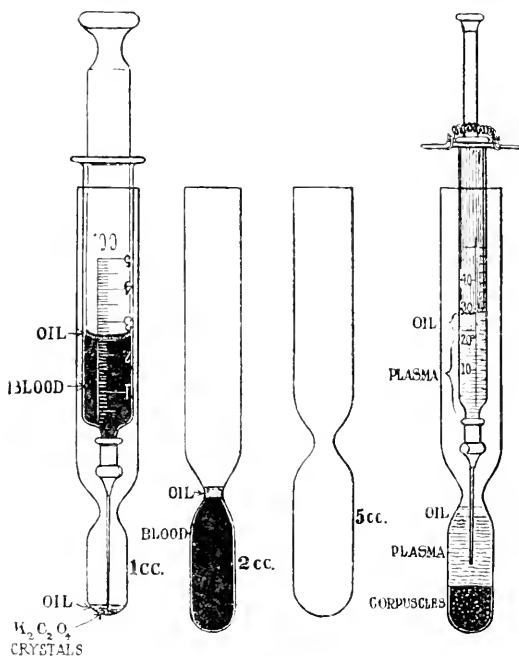


FIG. 1.

is at once delivered under oil into a centrifuge tube of special design (see Fig. 1). This tube is made of Pyrex glass³ and has at the bottom a bulb of 2 cc. capacity (30 mm. in length with an internal diameter of 11 mm. and a neck of 4 mm.). Tubes with

³ These tubes and other pieces of glass apparatus illustrated, exclusive of syringes, were made for us by the glass blowing department of Eimer and Amend.

bulbs of 1 and 5 cc. capacity have also been used, the latter being employed when a simultaneous estimation of the CO₂ content of plasma is to be made. 1 drop of neutral 20 per cent potassium oxalate is dried in the tube, after which 3 drops of mineral oil are added (6 drops in the case of the 5 cc. tube). In transferring the blood from the syringe to the centrifuge tube, the point of the needle is placed under the oil and sufficient blood delivered to bring the oil into the neck of the bulb. With the slight pressure

TABLE I.
Effect of Centrifugation under Oil on the pH of the Blood.

Case No.	First reading.	Initial pH.	Length of time of centrifuging.	Second reading.	pH value after centrifuging.	Remarks.
	<i>mm.</i>		<i>min.</i>	<i>mm.</i>		
1	29.0	7.21	5	29.0	7.21	Plasma first saturated with alveolar air.
2	33.0	7.26	5	33.0	7.26	" " "
3	45.5	7.43	5	45.5	7.43	Cells left in contact with plasma.
4	50.5	7.49	5	50.0	7.485	" " "
5	34.0	7.28	5	34.0	7.28	" " "
			10	34.0	7.28	" " "
6	43.0	7.40	5	43.0	7.40	" " "
			10	43.0	7.40	" " "
7	36.5	7.31	5	36.0	7.31	" " "
			10	36.5	7.32	" " "
8	39.5	7.35	5	39.5	7.35	" " "
			10	40.0	7.355	" " "
9	38.0	7.33	5	38.5	7.34	" " "
			10	38.5	7.34	" " "

exerted the blood readily takes up the oxalate and does not clot. The tube is centrifuged at moderate speed for about 2 minutes to separate the plasma.

As a check on the possible influence of oil on the hydrogen ion concentration due to centrifuging, the pH has been ascertained on specimens of blood centrifuged for 5 and 10 minutes, as well as for 2 minutes. In the case of the first two specimens the plasma was saturated with CO₂ at alveolar tension and the pH determined both before and after 5 minutes centrifuging. (The low initial pH in these two specimens is probably to be explained by

the fact that the plasma stood in contact with the cells some little time before separation.) The data given in Table I indicate that centrifuging under the conditions described causes no appreciable change in the pH.

Preparation and Preservation of Saline Solution.

A 0.9 per cent solution of sodium chloride in CO_2 -free water, to which has been added 10 cc. of 0.02 per cent phenol red solution⁴ for each 100 cc., is adjusted to a pH of between 7.4 and 7.5 with sodium hydroxide. This can conveniently be done by dipping the tip of a long slender stirring rod in 1 per cent sodium hydroxide, stirring into the solution in the flask, and repeating the operation until the solution, when placed in the cup of the colorimeter, gives the correct reading.

We have experienced considerable difficulty in keeping this extremely delicate solution at the correct pH, owing to a tendency for it to become acid. The apparatus, illustrated in Fig. 2, has, in a large measure, overcome this difficulty. The entire apparatus is constructed of Pyrex glass, an ordinary liter flask being the starting point. A cylindrical funnel, the stem of which has a small opening (1.5 mm.) passes almost to the bottom of the flask. When the glass stopper is removed, it is possible to fill the flask quickly with the saline solution. The stopper is inserted, the flask inverted, and any solution in the inverted funnel allowed to drain out. Finally, the funnel is wiped out and a tube containing soda-lime is tightly inserted. As an added precaution this tube is connected to a small wash bottle containing sodium hydroxide. The flask may also be filled through the stop-cock with the aid of suction, after the air in the flask has been freed of CO_2 by passing it through sodium hydroxide. We have found that when the saline solution is made up at a pH of about 7.55 (to allow for the slight drop in the pH, which we have been unable to prevent) it may be used for some time without adjustment.

⁴ This is made up as described by Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922, 81. 0.1 gm. of phenol red is ground in an agate mortar with 5.7 cc. of 0.05 N NaOH and made up to 25 cc. The 0.4 per cent solution is then diluted as needed to the 0.02 per cent strength with distilled water.

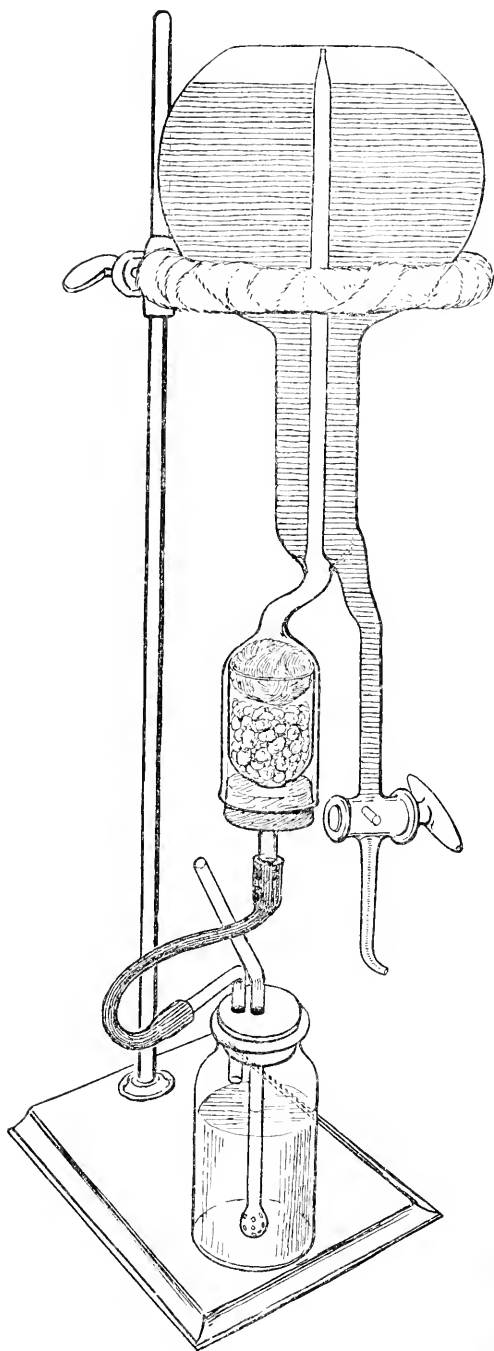


FIG. 2.

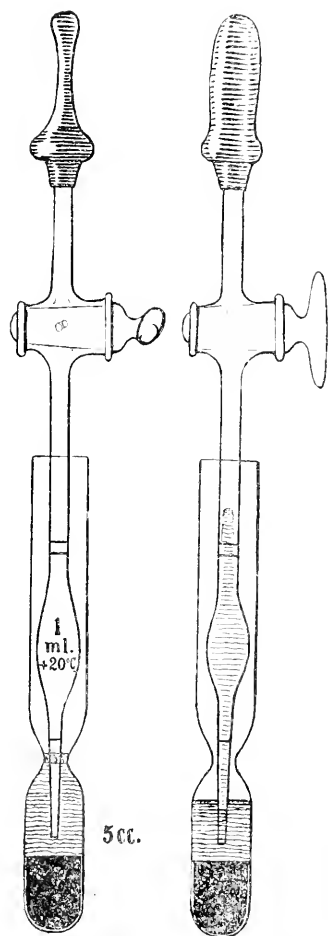


FIG. 3.

Colorimetric Measurement.

2 cc. of this saline solution are allowed to flow into the cup of the bicolorimeter under oil. A small portion of the separated plasma is now drawn into the 0.5 cc. tuberculin syringe (see Fig. 1), graduated in 0.01 cc. (the point of the needle can best be cut off), the air spaces of which are filled with oil. 0.1 cc. of the plasma is immediately discharged into the saline solution in the cup. This solution is stirred with a small glass rod and is then ready for color comparison.

Calibration of Wedges.

For this purpose the two wedges of the colorimeter are filled with Sørensen's buffer phosphate solutions, containing 2 cc. of 0.02 per cent phenol red for 20 cc. of phosphate solution, the front wedge having a pH value of 8.0 and the second wedge of 6.8. For the alkaline wedge 1.1 cc. of M/15 primary and 18.9

TABLE II.
Phosphate Mixture (Phenol Red Range).

pH	M 15 Na ₂ HPO ₄	M/15 KH ₂ PO ₄
	cc.	cc.
7.0	6.11	3.89
7.1	6.66	3.34
7.2	7.20	2.80
7.3	7.68	2.32
7.4	8.08	1.92
7.5	8.41	1.59
7.6	8.70	1.30
7.7	8.94	1.06
7.8	9.15	0.85

cc. of M/15 secondary phosphate are employed, while for the acid wedge 10.2 cc. of primary and 9.8 cc. of secondary phosphate are used. For the calibration of the wedges, nine different standards are employed covering the pH range from 7.0 to 7.8, the standards differing by 0.1 pH. The M/15 phosphate solutions should be prepared as described by Cullen² from special reagent salts (Merek's are satisfactory) by dissolving 9.47 gm. of the anhydrous Na₂HPO₄ in water and making up to a liter with distilled water. The primary potassium phosphate is similarly

prepared from 9.08 gm. of KH_2PO_4 . 5 (or 10) cc. portions of the different phosphate mixtures are prepared in Pyrex test-tubes according to the data given in Table II and 0.5 (or 1.0) cc. of the phenol solution is added to each tube. The readings made with the wedge containing the dominant color (alkaline, pH 8.0) are employed in plotting the curve from which the calculations are made. Although the solutions in the wedges apparently keep for a long time, we have made it a practice to check them against new standards once a week.

In the instances where the plasma is slightly cloudy the third wedge, containing water to which has been added 2 or 3 drops of milk and 1 drop of formaldehyde, is employed to equalize the field. In this way perfect color matches may always be obtained.

Calculation.

The factors worked out by Cullen² to correct the pH values to body temperature ($38^\circ\text{C}.$) have been employed. Most of our observations have been made at 24 or $25^\circ\text{C}.$ The following formula, using Cullen's factor of 0.22 as the average correction for colorimetric pH readings in human blood plasma, has been used:

$$\text{pH}_{38^\circ} = \text{pH}_t + 0.01 (t^\circ - 20^\circ) - 0.22$$

For example, a colorimetric reading of 42 has a pH value on our graph of 7.55. With a temperature of $24^\circ\text{C}.$ the formula would work out:

$$7.55 + 0.01 (24 - 20) - 0.22 = 7.55 - 0.18 = \text{pH } 7.37$$

Adaptation of Special Tubes to CO_2 Determination.

In cases where a pH determination of the blood plasma is indicated, it is generally desirable to ascertain the CO_2 content or CO_2 -combining power with the Van Slyke gas burette. With the aid of the 5 cc. tube illustrated in Fig. 1 it is possible to secure sufficient plasma for both determinations. In this connection we have found a special Ostwald-Folin pipette graduated to deliver 1 cc. between two points, in addition to the customary graduation, to be very convenient. To avoid disturbing the

corpuscles, we have also had such pipettes fitted with a glass stop-cock and a small rubber bulb of a capacity slightly greater than 1 cc. By compressing the bulb and turning the stop-cock sufficient suction may be secured to draw up the plasma while carefully watching the tip of the pipette (see Fig. 3). When the stop-cock is turned, the pipette can be conveniently handled.

Observations Made with the Method.

pH determinations with the method described have now been made on the blood plasma of more than 100 human cases. In a series of about twenty-five miscellaneous hospital cases, in which abnormal values for the pH were not anticipated, the figures varied between pH 7.35 and 7.43, with an average close to 7.39. The highest pH value so far obtained was 7.52 with a CO_2 -combining power of 87 and the lowest, a pH of 6.98 with a CO_2 -combining power of 12. Our observations will be discussed in a subsequent paper.

SUMMARY.

The colorimetric method of Cullen for the determination of the hydrogen ion concentration of blood plasma has been adapted to the bicolorimeter. By a simple technique contact of the blood with air is entirely excluded. The final determination is carried out on 0.1 cc. of plasma, and requires less than 10 minutes after the blood has been obtained. The error in color comparison falls within \pm pH 0.02.

THE EFFECT OF THERAPEUTIC APPLICATION OF EXTERNAL HEAT ON THE ACID-BASE EQUILIBRIUM OF THE BODY.*

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(Received for publication, June 25, 1923.)

The external application of heat as a therapeutic measure in the treatment of many types of disease has long found favor in the eyes of the medical profession. Despite the widespread use of this form of therapy in modern medicine, however, investigations recording the physiological changes that accompany such measures are far from complete. A clear understanding of the response of the body is desirable as a help in elucidating the pathological processes affected by such treatment and also for the purpose of applying this treatment more intelligently. It is common knowledge that the present wide and somewhat indiscriminate use of these measures is productive of harm as well as benefit.

Haggard (1), experimenting on himself, found a distinct lowering of the alveolar carbon dioxide tension after immersion for 20 minutes in a very hot water bath. As no corresponding fall in the CO₂-combining power of the blood was found, he suggested that a change in the reaction of the blood had taken place. Bazett and Haldane (2), likewise studying the effects of hot water baths, observed a marked hyperpnea and lowered alveolar carbon dioxide tension while the subject was in the bath. During this period, an alkaline urine was secreted containing much sodium

* The work here reported is part of a study on arthritis in collaboration with Robert B. Osgood, M.D., of Boston.

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bicarbonate and only traces of ammonia. Bazett and Haldane suggested that these findings were due to an acapnia following an increased sensitiveness of the respiratory center as a result of heat stimulation. This explanation seems reasonable in view of the almost identical changes in the composition of the alveolar air and urine following periods of forced breathing. These changes were reported by Davies, Haldane, and Kennaway (3), Collip and Bakus (4), and Grant and Goldman (5).

Pemberton, Hendrix, and Crouter (6), studying the effects of "electric bakes," observed a fall in the alveolar carbon dioxide tension during the bake with a rise above the initial value some time after its conclusion. They also found the oxygen percentage saturation of venous blood to be much higher during the bake. This presumably resulted from a greater blood flow which was induced by a quickened pulse. Pemberton and Crouter (7), in a continuation of these studies, observed an increased alkalinity of the urine and likewise a gradually increasing alkalinity or decreasing acidity of the sweat during the time of the exposure to heat. Since the present studies were completed, Koehler (8) has published the results of observations on the acid-base changes of the blood during acute fevers. Using the hydrogen electrode, he measured the pH of the blood of such patients and found an uncompensated alkalosis in a great majority of the cases. Koehler included in his studies observations on the reaction of the blood after a rise in body temperature induced by submersion in hot water baths. The blood of one of his three subjects changed in reaction from pH 7.39 to 7.65 during a half hour in a bath at 41-43°C. A marked fall in the total CO_2 content of the blood accompanied the change in pH. This subject showed distinct signs of tetany. In two other subjects, tetanic symptoms were successfully combated by breathing carbon dioxide.

Methods.

In these studies as in those previously reported from this laboratory, the heat was generally applied as a therapeutic measure. The source of heat was a portable electric cabinet. This cabinet was of such design that when the subject reclined on a bed he was covered from feet to shoulders by it. After 20 minutes in this cabinet, the temperature near to the skin of an individual was usually between 120 and 130°F. The "bakes" were continued for another 20 minutes so that the total exposure to heat was 40 to

50 minutes. The pulse rate, respiration rate, and oral temperature were taken at 10 minute intervals. Two subjects in this series of experiments were normal individuals. The others were adult men and women suffering from arthritis of various types and degrees of intensity.

Blood samples were obtained from the medial vein of the arm without stasis. Whenever the vein was sufficiently prominent, the usual preliminary use of a tourniquet was entirely dispensed with. The blood was allowed to flow, under oil, into a tube containing a small amount of neutral potassium oxalate. The first sample of blood was collected just before the beginning of the bake; the second when the electric cabinet had been removed, at a time when the body temperature and other effects of the external heat were at their maximum.

The carbon dioxide content, and oxygen content and capacity of these bloods were determined, usually in duplicate, using the method and apparatus (without the water jacket) of Van Slyke and Stadie (9).

Portions of each blood sample were pipetted into 300 cc. tonometers for exposure to desired tensions of carbon dioxide. The equilibration was effected in the following way. Calculated volumes of carbon dioxide were admitted from a gas burette into each tonometer. The tonometers were then rotated in a horizontal position in a water bath maintained at 37.5–38°C. At the end of 5 minutes the positive pressure within the tonometer was released by opening the stop-cock under water. The equilibration was considered complete at the end of 15 minutes. The tonometer was then held in an upright position to allow the blood to drain to one end. A slight positive pressure within the tonometer was induced by holding it under a hot water tap for a few seconds. The blood was allowed to flow into a pipette and was thus transferred to the Van Slyke apparatus and its CO₂ content determined. The exact composition of the gas phase in the tonometer was determined in the Haldane apparatus for gas analysis.

Two samples of each blood were exposed to different tensions of carbon dioxide, thus fixing two points on the absorption curve. From the data obtained CO₂ absorption curves were constructed. These curves represent completely oxygenated blood and in order to calculate from them the carbon dioxide tension and pH of venous blood, it was necessary to correct for the degree of oxygen unsaturation of the venous blood. Blood containing reduced hemoglobin unites with more carbon dioxide than does fully oxygenated blood. The ratio of the difference in CO₂ capacity between oxygenated and partly reduced bloods, to the degree of oxygen unsaturation of the reduced blood, has been quantitatively established by Doisy, Briggs, Eaton, and Chambers (10). We have used their ratio, or ordinate correction of 0.27 in locating the venous point from our curves. Pemberton, Hendrix, and Crouter (6), in a series of seventeen cases, studied the oxygen unsaturation of venous blood before and after heat exposure of the kind and duration described in the present experiments. We have used the average value for oxygen unsaturation of venous blood found by them in a number of our experiments where we did not record the oxygen content or capacity.

With the aid of the carbon dioxide absorption curves, then, the carbon dioxide tension of the venous blood was calculated. The hydrogen ion concentration, expressed as pH, was calculated from the $\text{NaHCO}_3:\text{H}_2\text{CO}_3$ ratio using Hasselbalch's equation and the value for pK_1 as 6.15. The recent work of Peters, Bulger, and Eisenman (11) on the variations of pK_1 in this equation indicates that the value 6.15, calculated by Van Slyke (12), is appropriate for use with human whole blood of normal hemoglobin content.

The pH of the bloods in four of the experiments was determined directly by the colorimetric method of Cullen (13). Changes in the alkali reserve as a result of the exposure to heat have been calculated as changes in the BHCO_3 content of the blood at a definite pH. Following the suggestion of Van Slyke, Austin, and Cullen (14) in their study of the acid-base changes consequent upon ether anesthesia we have compared the BHCO_3 content of the two bloods at the initial pH; that is, the pH of the blood before the heat application.

Results.

The results of the blood findings in fifteen studies, carried out in the manner described, are summarized in Table I. For a more detailed picture of the individual response and the analytical data obtained for construction of the CO_2 absorption curves the reader is referred to the protocols at the end of the paper.

With one exception only, we have observed an increased alkalinity of the blood following heat exposure. It will be seen that in a considerable number of experiments, the change in reaction of the blood is very marked, exceeding 0.1 of a Sørensen unit.

In Fig. 1 the CO_2 absorption curves of two experiments are presented graphically. They illustrate, respectively, the departure from normal where there is a marked change in blood reaction following the exposure to heat, and where the change in reaction and alkali reserve is slight.

The pH values obtained are not presented as necessarily absolute. Christiansen, Douglas, and Haldane (15) and others have shown that whole blood in the course of a few hours at room temperature develops quantities of a fixed acid sufficient to cause significant changes in its reaction. Such changes undoubtedly occurred in our experiments during the 3 to 5 hours required to complete the technical procedure incident to the equilibration and analysis. It is instructive in this connection to note that in Experiments 12 to 15 where the pH was determined by the direct method the low initial pH values were not found. The same in-

crease in the alkalinity of the blood was observed in this series of experiments as in the series where the pH was obtained from the $\text{BHCO}_3:\text{H}_2\text{CO}_3$ ratio. We feel that our results based as they are on a comparison of two bloods subjected to similar treatment satisfactorily measure in these respects the changes that follow the application of external heat.

TABLE I.

Changes in CO_2 Content, pH, and Alkali Reserve of Venous Blood After Exposure of the Body to External Heat.

Ex- per- iment No.	Subject.	CO ₂ content.		O ₂ content.		O ₂ capacity.		pH		BHCO ₃ at initial pH.		pH differ- ence.
		Before.	After.	Before.	After.	Before.	After.	Before.	After.	Before.	After.	
		vols. per cent	vols. per cent	vols. per cent	vols. per cent	vols. per cent	vols. per cent			vols. per cent	vols. per cent	
1	J.D.	46.2	43.7	17.3	21.2			7.26	7.52	42.9	48.1	0.26
2	S.H.	62.3	56.4	5.5	8.9	15.8	15.7	7.24	7.45	57.7	66.3	0.21
3	Dr.W.	58.0	50.9					7.21	7.39	53.4	67.6	0.18
4	S.R.	48.5	45.4	10.7	14.8	23.8		7.30	7.44	45.2	45.7	0.14
5	F.	60.9	55.1					7.23	7.36	56.2		0.13
6	A.L.E.C.	60.9	56.9					7.21	7.32	56.0	63.7	0.11
7	M.N.	55.6	54.7					7.21	7.28	51.1	55.0	0.07
8	G.C.	55.1	47.5					7.12	7.20	49.8	47.3	0.08
9	F.V.D.	52.2	43.1					7.20	7.25	47.9	41.9	0.05
10	B.L.	48.2	47.7	9.5	14.7	18.0	17.5	7.26	7.29	44.7	45.6	0.03
11	F.	54.7	57.1		6.7		16.2	7.27	7.24	50.7	43.1	-0.03
12	M.D.	54.2	53.1	13.2	15.1	18.7		7.43	7.55	51.5		0.12
13	B.G.	54.9	56.4	8.3	12.1	16.1	12.6	7.43	7.51	52.2		0.08
14	S.H.	54.6	51.3	9.3	13.5	19.5		7.42	7.47	51.8		0.05
15	M.G.	56.4	52.3	14.7	14.3	15.5	16.5	7.48	7.52	53.9		0.04

It will be seen from Table I that the total CO_2 content of the blood was lower after exposure to heat than before the heat was applied. At the same time the alkali reserve of the blood is somewhat greater. Examination of the oxygen capacity data in these experiments and in the series of seventeen cases studied by Pemberton, Hendrix, and Crouter does not reveal large or consistent variations between bloods drawn before and at the conclusion of the bake. Changes in the volume of the blood might follow the large loss of water in sweat during a heat experiment.

However, there is no indication of concentration of the blood if we accept the oxygen capacity of the blood as an index of blood volume changes. In Experiment 2, for example, there is no change

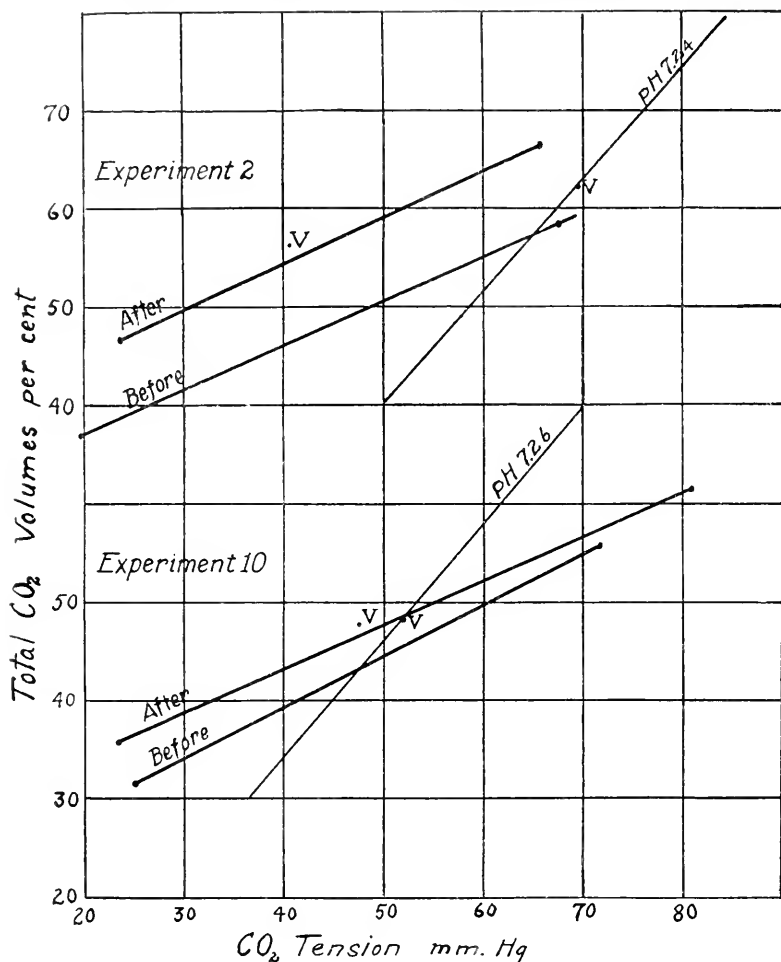


FIG. 1. CO_2 absorption curves of venous blood before and after the external application of heat.

in oxygen capacity although there is a distinct rise in alkali reserve. It would seem that this increase in alkali reserve is caused by a migration of base from the tissues into the blood.

The increased alkalinity of the blood is reflected in compensatory changes in the excretions of the body, similar to those observed by Bazett and Haldane. An alkaline wave usually can be detected in the urine, and the excretion of ammonia is diminished. The urinary alkaline wave may be transient and is frequently followed by a period when the urine is more acid than the urine collected beforehand. In experiments where we failed to detect an alkaline wave we usually had not secured a urine specimen promptly at the conclusion of the bake.

TABLE II.

Changes in the Composition of the Urine and Sweat After Exposure of the Body to External Heat.

Urine.						Forehead sweat pH.	
pH			Hourly NH ₃ - N.				
Before.	After.	1st hr. after.	Before.	After.	1st hr. after.	Before.	After.
			<i>mg.</i>	<i>mg.</i>	<i>mg.</i>		
6.8	7.4		17.4	12.0		7.5	8.0
7.0	8.0		33.6	18.0			
5.3	5.4		21.8	18.7		8.0	8.4
6.8	7.5		5.8	4.1		4.8	5.8
5.2	5.0		36.0	18.0		7.8	8.6
5.7	6.4	5.2	15.1	8.5	8.6	4.8	7.0
6.4	7.2	6.1	7.7	6.5	10.4	4.6	7.0

The progressive change in the reaction of forehead sweat from acid to alkaline, a change to which Pemberton and Crouter have already called attention, may be regarded as another compensatory sequel of the decreased acidity of the blood. In a further series of 75 observations, we have found that this change in reaction of sweat almost invariably accompanied the continuance of sweating during a bake. Typical results illustrating the extent of the changes in the composition of the urine and sweat are given in Table II.

In preliminary observations, Pemberton and Crouter (7) compared the reaction of the forehead sweat of a series of twenty-three arthritics and a series of ten normals during and after exposure to external heat. There were observed small differences

between these groups, the arthritic showing a slightly more acid reaction throughout. Extension of these observations to a series of forty-one arthritics and fourteen normals, however, reveals no difference in the average initial or subsequent reactions although some of the arthritics gave a pH lower than any normal showed. These last also seemed less subject to change during the bake and occasionally showed practically no change at all. The sweat reactions of the two groups are given in Table III.

TABLE III.

Changes in the Reaction of Forehead Sweat of Arthritics and Normals During External Application of Heat.

Normals.	Arthritics.	Arthritics.	Arthritics.
pH	pH	pH	pH
5.6-6.9	6.4-7.3-7.5	6.1-7.3-7.4	7.0-7.0
7.7-8.0-8.0	7.1-7.4-7.8	7.4-8.6	7.2-7.9-8.2
7.9-8.0-8.1	6.1-6.8	6.7-7.2	6.8-7.6
7.0-7.6-8.0	5.8-6.4	4.6-4.6	5.8-5.8-7.0
7.6-7.8	5.7-7.2-7.4	5.8-6.4-6.8	8.1-8.4-8.4
7.4-7.8-7.8	7.4-7.4	6.9-7.6-7.6	8.2-8.6-8.6
6.6-7.8-8.0	8.0-8.4-8.4	7.4-7.6-7.6	5.0-5.2-5.4
5.9-6.7-7.2	7.4-8.0	6.8-7.2-7.4	6.5-7.8-8.2
6.8-7.2-7.6	7.5-7.9	5.8-6.8-7.3	8.2-8.2-8.4
7.4-8.0-8.0	7.4-8.0-8.2	8.0- 8.2	5.7- 8.0
6.7-7.4-7.4	4.6-4.6-4.7	8.2- 8.2	4.6-6.0-7.0
5.8-7.0-7.1	6.0-7.6-8.0	7.3-8.0-8.1	7.5- 8.0
5.8-6.4-7.2	7.3-7.9-8.1	7.6- 8.0	6.3-7.6-7.6
7.2-7.8-7.8	8.0-8.4-8.4	8.1-8.2-8.2	
Average.			Average.
6.8-7.5-7.7			6.8-7.3-7.6

DISCUSSION.

The loss of carbon dioxide by the body during the heat experiments is undoubtedly of prime importance as a factor which leads to a greater alkalinity of the blood. The marked hyperpnea observed by Bazett and Haldane, and the fall in alveolar CO_2 found by them and other investigators, point to the conclusion that overventilation of the lungs results in a CO_2 deficit and a change in the reaction of the blood. In the same way extensively forced breathing produces similar changes in the blood reaction.

The analogy between the results of external heat experiments and the findings in overbreathing experiments must not be drawn too closely, however. Collip and Bakus, Grant and Goldman, and Henderson and Haggard (16), the latter authors working with dogs, report a large fall in CO_2 capacity of the blood as a result of overventilation of the lungs, a phenomenon we have failed to observe in our experiments.

The increased blood flow during a bake presumably favors a more ready gas exchange between tissues and blood. The skin is a path of CO_2 loss of some importance. Schierbeek (17) and, independently, von Willebrand (18), working in Tiegerstedt's laboratory, have found 1.4 gm. of CO_2 per hour given off by the skin during active sweating. Miscellaneous data on total CO_2 production taken from Lusk (19) indicate a total CO_2 production of about 30 gm. per hour with a 32 per cent increase during a rise in body temperature induced by a hot bath. If for the purpose of a rough calculation, we use these figures and those of Schierbeek and von Willebrand, 3 to 4 per cent of the total CO_2 lost during a period of raised body temperature and active sweating is eliminated by the skin.

Koehler, in discussing the alkalosis he observed in fevers, suggests that, with the increase of oxidation accompanying the rise in body temperature, the tissues suffer an oxygen want and that hydrogen ion stimulation of respiration results from the anoxemia. In our experiments as we have mentioned above, the venous blood is more highly saturated with oxygen after the application of external heat. It is difficult to conceive of an anoxemia arising in tissues bathed in this oxygen-rich blood.

It is rather surprising that changes in the acid-base equilibrium of the blood of the magnitude here reported should follow what is considered mild exposure to heat. The electric bake would seem to demand much less in the way of physical adjustments to it than is required during long continued very hot baths. In none of our experiments have we observed any serious distress or signs of tetany.

The disturbance of blood equilibrium that may follow the indiscriminate and extreme employment of heat in therapy, however, is clearly indicated from the response we have observed in mild bakes of short duration. There is here afforded some

explanation for the baneful effects which patients not infrequently experience at mercurial hands. It is not yet indicated that the response to heat by the arthritic is different from that of other individuals, at least in so far as it concerns acid-base equilibrium of the blood. Our observations on two healthy young men fail to indicate any difference between arthritics and normals in this respect.

SUMMARY.

1. CO₂ absorption curves have been constructed from the analytical and equilibration data obtained from venous blood drawn before and at the conclusion of 40 minutes exposure of the whole body to the heat of an electric cabinet. In other experiments the pH of venous blood was measured directly.

2. As a result of the external heat, the blood becomes more alkaline; there is a fall in its total content of CO₂ and a slight rise in alkali reserve. These changes in the acid-base equilibrium of the blood cause compensatory changes in the urine and sweat.

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Protocols.

Experiment 1.—J.D. Chronic arthritis. Duration of bake 37 minutes.
 Maximum temperature in cabinet 158°F.

	Initial.	Final.
Oral temperature.....	97.6°F.	99.0°F.
Respiration rate per min.....	14	20
Pulse rate per min.....	78	96
Urine pH.....	6.4	8.1
Forehead sweat pH.....	6.2	7.8

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	27.2	30.8
	62.2	54.5
Final blood.....	20.1	40.9
	96.1	65.8

Experiment 2.—S. H. Subacute arthritis. Duration of bake 40 minutes.
 Maximum temperature in cabinet 134°F.

	Initial.	Final.
Oral temperature.....	99.5°F.	100.6°F.
Respiration rate per min.....	16	18
Pulse rate per min.....	72	90
Urine pH.....	5.4	5.4

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	19.6	37.0
	67.4	58.3
Final blood.....	23.4	46.8
	65.7	66.2

Experiment 3.—Dr. W. Chronic arthritis. Duration of bake 50 minutes. Maximum temperature in cabinet 132°F.

	Initial.	Final.
Oral temperature.....	98.4°F.	99.8°F.
Respiration rate per min.....	16	12
Pulse rate per min.....	60	84
Urine pH.....	7.3	7.3
Forehead sweat pH.....	7.8	8.4

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	24.1	41.1
	82.2	59.4
Final blood.....	22.4	39.4
	65.7	62.5

Experiment 4.—S.R. Chronic arthritis. Duration of bake 45 minutes. Maximum temperature in cabinet 120°F.

	Initial.	Final.
Oral temperature.....	98.8°F.	99.8°F.
Respiration rate per min.....	20	16
Pulse rate per min.....	72	90
Urine pH.....	5.2	5.6
Forehead sweat pH.....	5.0	5.4

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	20.6	34.1
	90.5	62.3
Final blood.....	24.9	40.2
	73.8	57.7

Experiment 5.—F. Chronic arthritis. Duration of bake 48 minutes.
Maximum temperature in cabinet 118°F.

	Initial.	Final.
Oral temperature.....	98.2°F.	99.2°F.
Respiration rate per min.....	18	18
Pulse rate per min.....	72	78
Urine pH.....	5.4	5.2
Forehead sweat pH.....	4.6	5.0

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	34.0	51.0
	103.3	65.0
Final blood.....	40.0	49.2
	62.8	63.3

Experiment 6.—A.E.L.C. Normal. Duration of bake 32 minutes.
Maximum temperature in cabinet 126°F.

	Initial.	Final.
Oral temperature.....	98.1°F.	99.0°F.
Respiration rate per min.....	12	16
Pulse rate per min.....	72	84
Urine pH.....	5.6	7.2
Forehead sweat pH.....	6.2	7.3

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	21.6	36.7
	87.1	65.2
Final blood.....	22.1	40.4
	83.1	69.6

Experiment 7.—M.N. Subacute arthritis. Duration of bake 40 minutes. Maximum temperature in cabinet 138°F.

	Initial.	Final.
Oral temperature.....	98.6°F.	100.1°F.
Respiration rate per min.....	18	16
Pulse rate per min.....	72	96
Urine pH.....	6.0	6.8
Forehead sweat pH.....		8.0

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	24.0	38.2
	90.5	62.6
Final blood.....	22.4	40.8
	116.4	72.5

Experiment 8.—G.C. Normal. Duration of bake 30 minutes. Maximum temperature in cabinet 112°F.

	Initial.	Final.
Oral temperature.....	98.6°F.	99.1°F.
Respiration rate per min.....	12	14
Pulse rate per min.....	60	78
Urine pH.....	6.0	6.0
Forehead sweat pH.....	7.2	7.8

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	20.4	27.9
	79.5	53.6
Final blood.....	20.3	32.7
	83.0	55.8

Experiment 9.—F.V.D. Chronic arthritis. Duration of bake 42 minutes. Maximum temperature in cabinet 122°F.

	Initial.	Final.
Oral temperature.....	98.0°F.	99.3°F.
Respiration rate per min.....	12	10
Pulse rate per min.....	60	66
Urine pH.....	5.3	6.5
Forehead sweat pH.....	5.8	7.0

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	23.4	32.3
	70.5	53.1
Final blood.....	22.3	31.1
	81.9	56.3

Experiment 10.—B.L. Chronic arthritis. Duration of bake 47 minutes. Maximum temperature in cabinet 134°F.

	Initial.	Final.
Oral temperature.....	98.8°F.	99.4°F.
Respiration rate per min.....	16	12
Pulse rate per min.....	90	96
Urine pH.....	5.9	5.8
Forehead sweat pH.....	6.0	7.8

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	25.0	31.6
	71.5	55.7
Final blood.....	23.3	35.9
	80.9	61.2

Experiment 11.—F. Chronic arthritis. Duration of bake 30 minutes. Maximum temperature in cabinet 118°F.

	Initial.	Final.
Oral temperature.....	98.4°F.	99.1°F.
Respiration rate per min.....	16	12
Pulse rate per min.....	84	90
Urine pH.....	5.8	6.0
Forehead sweat pH.....	4.8	4.8

Equilibration.

	CO ₂ tension.	CO ₂ content.
	<i>mm.</i>	<i>vols. per cent</i>
Initial blood.....	17.6	32.7
	79.7	64.3
Final blood.....	17.4	29.3
	75.9	61.3

Experiment 12.—M.D. Chronic arthritis. Duration of bake 50 minutes. Maximum temperature in cabinet 130°F.

	Initial.	Final.
Oral temperature.....	97.8°F.	100.3°F.
Respiration rate per min.....	20	16
Pulse rate per min.....	60	84
Urine pH.....	7.0	8.0

Experiment 13.—B.G. Chronic arthritis. Duration of bake 50 minutes. Maximum temperature in cabinet 130°F.

	Initial.	Final.
Oral temperature.....	98.0°F.	100.4°F.
Respiration rate per min.....	12	16
Pulse rate per min.....	66	102
Urine pH.....	6.4	7.2

Experiment 14.—S.H. Subacute arthritis. Duration of bake 65 minutes. Maximum temperature in cabinet 124°F.

	Initial.	Final.
Oral temperature.....	99.0°F.	101.0°F.
Respiration rate per min.....	16	20
Pulse rate per min.....	72	90
Urine pH.....	5.3	5.3
Forehead sweat pH.....	6.2	8.2

Experiment 15.—M.G. Subacute arthritis. Duration of bake 30 minutes. Maximum temperature in cabinet 110°F.

	Initial.	Final.
Oral temperature.....	98.2°F.	99.5°F.
Respiration rate per min.....	12	16
Pulse rate per min.....	84	90
Urine pH.....	6.2	7.8
Forehead sweat pH.....	5.7	5.8

THE DETERMINATION OF FIBRINOGEN BY PRECIPITATION WITH SODIUM SULFATE COMPARED WITH THE PRECIPITATION OF FIBRIN BY THE ADDITION OF CALCIUM CHLORIDE.

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Recently we have had occasion to make a considerable number of determinations of fibrin by the method of Cullen and Van Slyke, which we have modified slightly (1). Simultaneous determinations were made by precipitating fibrinogen with 10.6 per cent sodium sulfate at 37°C., and some determinations by the methods of Gram (2) and of Foster and Whipple (3). The fundamental procedures of various recent methods for the determination of fibrin and fibrinogen are as follows:

(a). Cullen and Van Slyke (4). The fibrinogen of oxalated or citrated plasma is permitted to clot by the addition of calcium chloride. The nitrogen content of the fibrin is determined after suitable washing.

(b). Howe (a). This procedure is identical in part with the above, but modified by the use of smaller quantities of plasma, a greater dilution during coagulation, and the analysis of an aliquot of the filtrate. The two methods give essentially similar results.

(c). Gram. Procedure similar to that of Cullen and Van Slyke so far as the coagulation is concerned, but differs in that the fibrin is washed with water, dehydrated with alcohol and ether, and weighed directly.

(d). Foster and Whipple. Very similar to the procedure of Gram, but differs in that the fibrin is not dehydrated with alcohol and ether but is dried at 110°C., weighed, ashed, again weighed, and the fibrin calculated by difference.

(e). Wu (5). Identical with the procedure of Cullen and Van Slyke, but modified in that the fibrin is analyzed by a colorimetric procedure. No attempt has been made to use this method; for some purposes it should be very valuable.

(*f*). Howe (*b*). Precipitation with 10.6 per cent sodium sulfate at 37°C . This procedure has not been described, but data obtained with it have been published (6). It is an extension of the method proposed for the determination of the proteins of blood (1). It is not necessary to use sodium sulfate. The following molecular concentrations (grams of salt in a liter of water) of other salts will give essentially the same results—1.0 molar lithium sulfate; 1.25 molar magnesium sulfate; 0.9 molar, with regard to the phosphate radicle, of a mixture of mono- and disodium phosphates in the proportion of 1:2; 1.125 molar solution of a similar mixture of potassium phosphates; or a 3.75 molar solution of sodium chloride. The procedure for sodium sulfate is as follows: Pipette 0.5 cc. of oxalated or citrated plasma into a suitable test-tube, add 15 cc. of 11 per cent sodium sulfate solution¹ at 37°C . After the precipitate has settled,² or better after 3 hours, filter on a dry filter, and take 5 cc. of the filtrate for analysis according to any suitable micro nitrogen method.

In some of our work determinations have been made by a number of different procedures, but the data presented concern chiefly a comparison of procedures (*b*) and (*f*). Following the appearance of the procedures of Gram and of Foster and Whipple, determinations were made by these methods, particularly by a combination of the two methods in which the fibrin was removed after coagulation according to Foster and Whipple and dehydrated according to Gram. The data presented are of interest in that essentially the same results were obtained by methods which involve apparently different types of reaction for precipitation.

EXPERIMENTAL.

A series of 80 determinations on different samples of plasma according to procedure (*f*) was compared with a similar series according to procedure (*b*). Data according to (*b*) were taken as the basis of comparison. In the course of the analytical work when the results by the two methods did not agree the determination by procedure (*b*) was repeated. We are, then, comparing in most cases verified values by method (*b*) with unverified values by method (*f*). The total protein nitrogen content of the plasmas

¹ The volume of 11 per cent sodium sulfate used when added to the 0.5 cc. of plasma gives a final concentration of approximately 10.6 per cent of sodium sulfate, or a 0.75 volume-molecular solution.

² The precipitate as it first forms has the appearance of fine needle-like crystals or of finely divided metal in gasoline. This form soon changes into a flocculent precipitate. This phenomenon has not been observed with any other protein fraction, where the precipitation of the first fraction occurs, *e.g.* euglobulin in serum or pseudoglobulin II in new-born calf serum.

ranged from 0.60 to 1.39 gm. of nitrogen per 100 cc. of plasma, and the fibrin values from 0.028 to 0.233 gm. of nitrogen per 100 cc. of plasma. The high total nitrogen and high fibrin values did not necessarily coincide. Furthermore, we are comparing results for fibrin with those for fibrinogen. Data on all samples analyzed as indicated are included, there was no selection of material.

An analysis of the data shows the average variation of results by the sodium sulfate precipitation from those of the calcium chloride coagulation to be 0.004 gm. of nitrogen per 100 cc. of plasma, in favor of the sodium sulfate precipitation. If we eliminate the determinations, fifteen, which showed a variation greater than 0.04 gm. of nitrogen per 100 cc. of plasma, the average variation is 0.0004 gm. of nitrogen. The distribution of the plus and minus differences is nearly even, with a tendency in favor of the sodium sulfate precipitation. In forty-one cases the quantity of nitrogen precipitated by sodium sulfate was greater than by calcium chloride, and in thirty-one cases it was the reverse, while there were eight cases in which the amount of protein nitrogen was the same. From the data we have concluded that the results obtained by the determination of fibrinogen by precipitation with 10.6 per cent sodium sulfate at 37°C. are comparable with those obtained by the coagulation of fibrinogen with calcium chloride from citrated plasma at room temperature. It is to be remembered that there is a similarity in the two procedures in that in both cases the filtrate was analyzed and the result subtracted from the same value for total nitrogen.

In the experiments on the weight of fibrin obtained from plasma as compared with the determination of nitrogen, it was found by preliminary determinations that the results by the Gram method were the same as those of the Foster-Whipple procedure within the limits of error of the methods. In subsequent work, therefore, certain details from each procedure were used, as follows: 2 cc. of plasma were measured accurately, to this were added 40 cc. of 0.8 per cent NaCl and 2 cc. of 2.5 per cent CaCl_2 . After the fibrin had formed it was removed according to the procedure of Foster and Whipple. From this point the fibrin was dehydrated with alcohol and ether and weighed according to the technique of Gram. The solution remaining after the removal of the fibrin was filtered on a dry filter and analyzed according to procedure

(b) above. For comparison with these results the fibrin formed in the determination by procedure (b) from 0.5 cc. of plasma was similarly removed and treated and the solution analyzed.

A series of thirteen plasmas was analyzed in this manner. The total nitrogen varied from 0.804 to 1.445 gm. of nitrogen per 100 cc. of plasma, and the fibrin from 0.047 to 0.155 gm. of nitrogen per 100 cc. of plasma. The averages of the determinations for the 0.5 and 2 cc. samples, expressed with relation to 100 cc. of plasma, were 0.106 and 0.105 gm.; weight of fibrin 0.658 and 0.659 gm. These results give 16.1 and 15.95 per cent, respectively, for the weights of fibrin obtained from 0.5 and 2 cc. samples. The percentage values are given only as an indication that the relation between the nitrogen determined and the weight of the fibrin found is within reasonable limits, for the quantities of plasma used are too small for any other deduction.

DISCUSSION AND CONCLUSIONS.

From the figures presented it appears that any one of the procedures proposed for the determination of fibrin or fibrinogen is satisfactory. The significance of the data is not confined to the relative accuracy of the procedures nor to the development of a new method. The Cullen-Van Slyke method, or our modification of this method, is in many ways preferable to the use of sodium sulfate at 37°C . when it is desired to determine fibrin nitrogen, because it can be carried out at room temperature. If fibrin alone is to be estimated, the combination of the Foster-Whipple and Gram methods is preferable. The fact that 10.6 per cent sodium sulfate, an approximately 0.75 volume-molecular solution, yields essentially the same results as the formation of fibrin from fibrinogen by the addition of calcium chloride to citrated plasma is of importance. This fact brings the determination of fibrinogen into harmony with the other concentrations of sodium sulfate used in estimating proteins (1). Thus it appears that the concentration of sodium sulfate required for the precipitation of the various "protein fractions" of blood after the first fraction is obtained is 0.25 molar. The concentrations of sodium sulfate which yield the various fractions are: fibrinogen 0.75 molar (10.6 per cent); euglobulin, 1.00 molar (14.2 per cent);

pseudoglobulin I, 1.25 molar (17.7 per cent); and pseudoglobulin II, 1.50 molar (21.5 per cent).³ The first two fractions give results which agree very closely with those obtained by other procedures in which the precipitating factor is not that of "salt-ing out", as the term is ordinarily used; *i.e.*, fibrin by recalcification and euglobulin by dilution and acidification with carbon dioxide.

The fact that similar results were obtained for fibrin by the two procedures used also indicates that the "overlapping" of precipitation limits or contamination of precipitates by adsorption is approximately the same in either process. This conclusion is reached from a consideration of the different bloods used; it did not matter whether the blood was rich or poor in fibrin or contained none of the next protein fraction, new-born calf, or was rich in euglobulin.

There may be some question as to the propriety of comparing results for the determination of fibrin with those for fibrinogen.⁴ The question of the presence of Hammarsten's fibrinoglobulin (8) has not been entirely settled. The analyses of the blood plasma and serum of new-born calves suggest that there is not a protein present which requires a salt concentration for precipitation other than that required for fibrinogen, or if it is present it is not in readily demonstrable amounts. Thus in the plasma a precipitate is completed at 10.6 per cent of sodium sulfate and there is not a further appreciable precipitate until after 17.7 per cent of sodium sulfate has been added, and in serum no appreciable precipitate occurs until after 17.7 per cent of the salt is present. In the latter case euglobulin is not present to interfere with the observation by a possible overlapping of precipitation limits. The similarity of the results by the various methods outlined above is suggestive in this connection.

³ The concentrations for euglobulin and pseudoglobulin I given are slightly different from those first proposed, 13.5 to 14.5 and 17.4 per cent of sodium sulfate, respectively. The concentrations given above were found to give the same results with blood serum and are the optimum concentrations for colostrum (7).

⁴ That fibrinogen was present in precipitates with salt solutions was demonstrated by dissolving the precipitate in water and adding serum; fibrin was formed.

nitrogen precipitated by a given concentration of sodium sulfate as compared with other procedures which are often considered as evidence of protein fractions of blood, then the data obtained with the concentrations of sodium sulfate given are indicative of the completion of precipitation of fibrinogen, euglobulin, pseudoglobulin I, and pseudoglobulin II, respectively, present in blood plasma. From a qualitative point of view the constancy of the quantitative results obtained by the addition of a definite amount of a salt under definite conditions of temperature and dilution does not necessarily indicate a separation of one protein fraction from another, since any concentration of salt will under such conditions give concordant results with the same sample of serum or plasma. It is because of the latter fact that the critical zones are of importance.

There is a regularity in the increase in the amount of sodium sulfate required for the precipitation of each succeeding protein fraction from blood serum or plasma, 0.25 mol, after the first protein fraction (fibrinogen) is precipitated, which appears to have a bearing on the nature of the salting-out process and the separation of proteins by such procedures. If the series of concentrations of sodium sulfate at 37°C. is extended by 0.25 mol beyond 1.50 mol¹ (total globulin) into the region of concentrations of salts required to precipitate albumin, it has been found that with a 1.75 molar solution a relatively small quantity of protein is precipitated, of a magnitude approximately that precipitated between a 1.25 and a 1.50 molar solution. With a 2.00 molar solution of sodium sulfate a larger precipitation occurs and another large precipitation with 2.25 molar solution. A 2.50 molar solution of sodium sulfate precipitates practically all of the protein, and with a 2.75 molar solution the precipitation of protein is complete. There is a possible significance in the fact that the largest albumin fraction in horse serum and egg white occurs between 1.75 and 2.00 mols of sodium sulfate, while in cow serum the largest fraction is between 2.00 and 2.25 mols of sodium sulfate. Horse serum and egg white contain readily crystallizable albumin, while it is difficult to obtain crystalline albumin from cow serum. The serum of the chicken and the pig show a partition of albumin

¹ Unpublished results.

more like that of horse serum, and the serum of the sheep and man like that of the cow.

The data with regard to the regularity of increase in the concentration of salt required to precipitate succeeding globulin fractions have been extended to other salts. This has been accomplished by comparing the results obtained with the concentrations of sodium sulfate given above with those of various concentrations of lithium sulfate, magnesium sulfate, potassium sulfate, mixtures of sodium and potassium phosphate, sodium chloride, potassium chloride, and lithium chloride. Calcium chloride and magnesium chloride have been studied with various results. The data obtained have a significance which is independent of whether or not the quantitative data obtained represent certain protein fractions.

EXPERIMENTAL.

Determinations were made of the nitrogen contained in the filtrates obtained from the precipitation of protein fractions from 0.5 cc. of blood serum or plasma by the addition of 15 cc. portions of various concentrations of salt, calculated on the anhydrous basis; a new portion of blood was taken for each determination. The concentrations of salt actually used were higher than those given in the tables by an amount equal to that required to bring the 0.5 cc. of plasma to the same concentration. The general procedure of precipitation and filtration has been previously described (4). All precipitations were made at 37°C. although it has been found in cases where the salt was sufficiently soluble at ordinary temperatures that similar results may be obtained at room temperature. The data were obtained from time to time on various samples of blood as they became accessible in other connections and they represent results on fresh blood. On the other hand, samples of serum which had been kept for 8 to 10 months gave similar values. The results presented represent concentrations of a given salt required to yield the analytical values obtained with sodium sulfate.

In Table I are summarized data with regard to the different salts.

The following facts are evident from the data presented in Table I:

(a). The molar concentration of a salt required for the completion of precipitation of the first protein fraction may be different for different salts.

(b). There is a constant additional amount of salt required for each succeeding fraction after the precipitation of the first fraction, called the "increment of salt."

TABLE I.

*Volume-Molar Concentrations of Salt Required for the Equal Precipitation of Certain Protein Fractions from Blood When Compared with the Results Obtained with Sodium Sulfate.**

Salt.	Protein fractions.				Increment of salt.	
	I	II	III	IV	Mols.	Equivalent of base.
Sodium sulfate.....	0.75	1.00	1.25	1.50	0.25	0.50
Potassium ".....	0.75					
Lithium ".....	1.00	1.40	1.80	2.20	0.40	0.80
Ammonium " †.....	1.25	1.50	1.75	2.00‡	0.25	0.50
Magnesium ".....	1.25	1.625	2.00	2.375‡	0.375	0.75
Zinc " §.....	1.25	1.50	1.75	2.00	0.25	0.50
Sodium phosphate mixture.....	0.90	1.20	1.50	1.80	0.30	0.50
Potassium phosphate mixture.....	1.125	1.425	1.725	2.025	0.30	0.50
Sodium chloride.....	3.75	5.00‡			5 × 0.25	5 × 0.25
Potassium ".....	3.75					
Lithium " ¶.....	5.00	6.00	7.00	8.00	5 × 0.20	5 × 0.20

* No data are given for *magnesium* and *calcium chloride*. If the relation between sulfates and chlorides holds for these two salts, it is impossible to have sufficient magnesium chloride in solution to precipitate the first fraction from plasma. The concentration of calcium chloride required for serum is of the same order of magnitude as that for the chlorides studied. A 5 molar solution of calcium chloride when added to cow serum will give a slight precipitate, but will not precipitate new-born calf serum. If the solutions obtained be allowed to stand, ultimately most of the protein will be precipitated in both cases. These precipitates are insoluble upon the addition of water. It is apparent that in the case of calcium chloride there is a secondary action which is not related specifically to Fractions II or III and which is apparently a general protein reaction.

† The values for ammonium sulfate are not so clear-cut as for the other salts. The data were obtained by determining the protein by the pro-

(c). The increment of salt required for various salts may be different, but is not necessarily so.

(d). The increment of base required for each fraction appears to be the same, or a multiple, for different anions; *i.e.*, there appears to be a definite relation between the various series of salts.

The Influence of the Cation in the Precipitation of Protein.—In Table II data are presented showing the effect of varying the proportions of monosodium (or potassium) and disodium (or potassium) phosphate. The proportions of the two phosphates selected were those used by Sørensen in preparing dilute phosphate solutions having different hydron concentrations. The hydron concentrations of the solutions used at the given salt concentrations are not those obtained in dilute solution; they did give the correct hydron concentration when diluted.

It is apparent from the data presented in Table II, which cover only extreme combinations of phosphates, that:

(a). Within a limited range, variations in the relative proportions of monosodium (or potassium) and disodium (or potassium) phosphate, equal precipitation of protein occurs when the concentration of base is the same.

(b). When the concentration of the cation is constant the

cedure of Wu (5). These values agree closely with those which have formerly been used on the basis of percentage saturation. The analytical values on cow serum obtained with ammonium sulfate agree with those obtained with sodium sulfate. On the other hand, when a concentration for Fraction III is added to new-born calf serum a slight precipitate is obtained with ammonium sulfate, but not with any of the other salts used. No precipitation occurs at 1.58 mols of ammonium sulfate. This is the only case, so far found, in which the absence of precipitation with new-born calf serum has not coincided with the agreement of analytical data on cow serum. We prefer for the present to assume that there is an anomalous behavior in the case of ammonium sulfate.

‡ The following are the approximate values in terms of a saturated solution for the salts indicated: ammonium sulfate, 2.00 molar = one-half saturated solution; magnesium sulfate, 2.375 molar = saturated solution; sodium chloride, 5.00 molar = saturated solution.

§ Precipitations with zinc sulfate are not so clear-cut as with the other sulfates; *i.e.*, it is much more difficult to obtain consistent determinations.

¶ It is possible to obtain consistent data with lithium chloride only by filtering soon after precipitation, otherwise the precipitate becomes insoluble and additional protein is also precipitated.

concentration of the PO_4 radicle may vary nearly 0.8 mol without appreciably affecting the analytical results. This fact holds for all the globulin fractions.

(c). The increment of base between the various protein fractions is the same as that for the sulfates. The data presented on mixed salts, given below, makes it permissible to assume that if potassium sulfate were sufficiently soluble it would give results similar to those obtained for sodium sulfate.

(d). The beginning concentration for the precipitation of the first protein fraction, with regard to the PO_4 ion, is not the same

TABLE II.

Precipitation of Protein by Various Mixtures of Potassium Phosphate. Results Are Expressed as Grams of Nitrogen per 100 Cc. of Plasma and Represent the Quantity of Nitrogen Remaining in the Filtrate after Precipitation.

Salt.	Protein fractions.*			
	I	II	III	IV
Sodium sulfate.....	0.940	0.791	0.621	0.524
$\frac{\text{KH}_2\text{PO}_4}{\text{K}_2\text{HPO}_4}$ ratio.				
$\frac{1}{15}$	0.912	0.795	0.625	0.524
$\frac{1}{2}$	0.932	0.791	0.620	0.522
$\frac{8}{1}$	0.930	0.783	0.593	0.520

* Total nitrogen = 0.993 gm. N.

for the sodium and potassium phosphates as it is for the sulfates and chlorides.

(c). There is a slightly greater precipitation of protein with the higher proportions of acid phosphate, but this is small in comparison with the quantity of protein precipitated. If the proportion of acid phosphate be sufficiently increased, a precipitation occurs which is not related to the concentration of base but to the hydron concentration.

Table III contains data relating to the variation in the concentration of PO_4 ion which exists when the concentration of base is maintained the same.

Precipitation by Mixtures of Salts.—The regular increment required for the precipitation of the various protein fractions after

the concentration for the first fraction has been determined offers an opportunity for comparing mixtures of salts. Some experiments have been made on the assumption that the same amount of protein will be precipitated by an increment of salt whether it be added, alone or in multiples, to its own beginning concentration or

TABLE III.

*Molecular Concentrations with Regard to PO_4 to Give Equal Na or K Concentrations for Sodium or Potassium Phosphate Equivalent to the Na or K Concentration at pH 7.0.**

	Protein fractions.			
	I	II	III	IV
Sodium phosphate mixture.				
Concentration of sodium, mols.....	1.50	2.00	2.50	3.00
Mols of PO_4 ion. $\frac{\text{NaH}_2\text{PO}_4}{\text{Na}_2\text{HPO}_4}$ ratio.				
$\frac{8}{1}$	1.35	1.80	2.25	2.70
$\frac{1}{2}$	0.9	1.20	1.50	1.80
$\frac{1}{16}$	0.79	1.059	1.32	1.58
Potassium phosphate mixture.				
Concentration of potassium, mols....	1.875	2.375	2.875	3.375
Mols of PO_4 ion. $\frac{\text{KH}_2\text{PO}_4}{\text{K}_2\text{HPO}_4}$ ratio.				
$\frac{8}{1}$	1.6875	2.1375	2.5875	3.375
$\frac{1}{2}$	1.125	1.425	1.725	2.025
$\frac{1}{16}$	0.936	1.223	1.481	1.739

* The ratios of monosodium (or potassium) phosphate to disodium (or potassium) phosphate used when in 0.15 molar solution have hydron concentrations as follows: $\frac{8}{1}$ —pH 5.8; $\frac{1}{2}$ —pH 7.0; $\frac{1}{16}$ —pH 8.0.

to the beginning concentration of another salt. Preliminary data indicate that for mixtures of sulfates and of sulfates and chlorides the assumption is in general correct, or the deviations are not great. Mixtures of phosphates and sulfates have given unsatisfactory results so far, but not sufficient to be called negative.

DISCUSSION.

The data presented above bear upon an old problem. The first related study was made by Lewith (6), which was amplified and discussed by Hofmeister (7). The subject has been discussed in detail by Spiro (8) and Chick and Martin (9), reviewed by Höber (10) and Robertson (11), and therefore another attempt will not be made here.

These experiments are comparable to those of Lewith and of Hofmeister in that the concentration of salt required for the completion of the precipitation of the first fraction should correspond very closely with the beginning of visible precipitation of protein which was used by these investigators as the index of the relative efficiency of different salts. Our index of equal efficiency is, however, more rigorous. The number of salts studied was smaller than might be desirable. With few exceptions no attempt has been made to study salts which are not capable of precipitating more than two of the globulin fractions. It is possible that with the extension of the suggestive results obtained with mixed salts we have a method of studying the relative precipitating efficiency of other less soluble salts. The data presented apply particularly to the comparison of the relative effects of the cations. Changes in hydrion concentration have not been studied except such as were present in the phosphate mixtures; the actual hydrion concentration in these cases is only known approximately. The precipitations took place in a range of hydrion concentrations in which considerable variations can occur without affecting the quantity of protein precipitated (9, 12, and our own experiments).

The results obtained have both a practical and a theoretical value. From a practical point of view, which is related entirely to the current acceptance of protein fractions obtained by salting-out, the number of salts which may be used for fractioning and studying the proteins of blood has been extended.

It is impossible to do more than to speculate on the theoretical significance of the data at this time, since an adequate conception of the nature of concentrated solutions is not available. Past evidence combined with that which is presented makes it apparent that the precipitation of protein fractions by means of salts under the conditions employed involves (*a*) both the cation and the

anion of the salt, (b) for a given anion the cation is the determining factor in precipitation, (c) the valence of the cation is not a predominant factor, and (d) for a given anion the order of magnitude of the concentration of the cation required is a multiple of that required for that of any other anion and is characteristic of the anion. The latter statement holds in general for the salts studied.

The relative efficiency of salts can be compared on two bases: (a) that used by Hofmeister in which we would use the completion of precipitation of the first protein fraction from plasma, and (b) the increment of salt or of cation required for each subsequent fraction. Our results agree in general with those of Lewith and of Hofmeister, with the exception of lithium sulfate and potassium phosphate which are required in higher concentrations than found by these authors. The determination of the concentration of salt required for the precipitation of the first fraction from blood plasma is open to some variation in case only that concentration is being determined. This is true because there is a zone, provided the concentration of the immediate protein fractions is not too large, of at least 1 per cent of salt in which there may be but slight change in the quantitative results. When the concentrations for the subsequent fractions are determined, however, this latitude is restricted since four points must be satisfied instead of one.

The quantity of chloride required for precipitation of the various fractions is of the order of five times the beginning concentration for the corresponding sulfate. This relation holds for sodium chloride, potassium chloride, and lithium chloride, and apparently for calcium chloride. If we assume that the quantity of magnesium chloride required to precipitate the first fraction is five times the concentration for magnesium sulfate then it is not possible to have sufficient magnesium chloride in solution to precipitate the first fraction; magnesium chloride does not precipitate the first fraction of blood. On the other hand, the addition of five times the increment of magnesium ion found for magnesium sulfate to a 0.75 molar solution of sodium sulfate will not give a precipitate with serum; a good precipitate is obtained with a solution containing 1.375 mols of sodium sulfate plus 1.875 mols of magnesium chloride.

When the various salts are compared on the basis of the increment between the various fractions after the precipitation of the first protein fraction of plasma, we find that for the sulfates, sodium and potassium are equally effective, magnesium is next, and then lithium. The correspondence of the values for sodium and potassium may be of significance. Brönsted (13) has found the salting-out capacity of sodium and potassium ions to be very nearly equal, approximately 1 per cent higher for sodium than for potassium. Such a difference would not be readily demonstrable in our work; if it was present, we might still be able to obtain concordant results at all of the critical zones although a long series of experiments should show a deviation in one direction.

The relation between the increment of salt required for the succeeding fractions for the sulfates and chlorides does not seem to hold as regularly as for the beginning concentrations. With the exception of sodium and potassium chlorides, there are difficulties in working with the available chlorides as indicated in the experimental work. For sodium and potassium chloride the increment is five times the increment found for the sulfates. For lithium chloride the increment is five and one-half times the increment for lithium sulfate. With regard to the increment of the equivalent of base the values for the chlorides are two and one-half times the value for the sulfates in the case of sodium and potassium and one and one-quarter times the value for lithium. In the case of magnesium chloride the increment is apparently much larger than five times the increment for magnesium sulfate. The data cited with regard to the precipitation of the first protein fraction of blood serum by magnesium chloride and magnesium sulfate indicate that there is a much larger difference or that we were observing another phenomenon of mixed salts. On the other hand, there is no difficulty in mixing sodium sulfate and lithium or magnesium sulfate or of mixing sodium chloride and sodium or magnesium sulfate.

A relation has been sought by various investigators—see Washburn (14) and Höber (10) for a consideration of such work—between the hydration of the ions of a salt and its relative efficiency in salting-out, which has not always been found. From the data available on the hydration of the ions of salts and the results presented in this paper, it is apparent that so far as the relation

existing in the solution, exclusive of the protein, is concerned salts with highly hydrated ions are not so effective as those with less hydrated ions.² Thus the lithium ion is more highly hydrated than the sodium ion (15, 16) but sodium sulfate and sodium chloride are more effective in precipitating proteins than lithium sulfate and chloride, when compared on the basis of the beginning concentration. Potassium and sodium sulfates or chlorides are approximately equally effective, but the potassium ion is less hydrated than the sodium ion.

The recent work of Garrett and Lewis (17) indicates that the assumption that the water of hydration is always reserved for the exclusive use of the substance hydrated is open to question. These investigators found that the solvent power of the water of hydration of various substances may vary. They have suggested that "when both colliding individuals are hydrated, mutual solubility or penetration occurs. If only one individual is hydrated no generalization can as yet be made."

The data on the precipitation of proteins by mixtures of sodium or potassium phosphates indicate that for a given anion the cation is the effective agent. With essentially equal precipitation when the concentration of the cation was kept constant, the variation in the concentration of the PO_4 ion was sufficient to have precipitated practically all of the globulin present in blood serum, on the assumption that the cation and anion are equally effective. That the anion has an effect in the precipitation of protein is evident from data on the relative effectiveness of various anions when the same cation is present.

² It is possible that in the case of the high concentrations of the chlorides of lithium and calcium there is a relation between the relative ease with which these compounds produce insoluble protein precipitates and their pronounced tendency to form hydrates. Thus protein precipitated by lithium chloride is soluble in water, plus the salt present, for some time after precipitation but ultimately it becomes insoluble. The precipitate with lithium sulfate does not become insoluble within a reasonable length of time. With calcium chloride the action is much more rapid, if the concentration of calcium chloride is just sufficient to produce a good turbidity the precipitate can be redissolved by the addition of water for 2 or 3 hours afterward, but ultimately this is impossible. On the other hand, if a good flocculation is produced with calcium chloride (higher concentration), it is necessary immediately to add water to prevent the formation of an insoluble precipitate. The change appears to be progressive.

While data have many times been presented with regard to the specificity of action of ions in the salting-out of protein by the comparison of the effects of various cations having a common anion, so far as we can find no one has previously demonstrated that under certain conditions the action of one ion is relatively independent of the other by varying the quantity of one ion while the other ion is kept at the same concentration.

Sodium and potassium have been found to be equally effective as precipitants of protein with regard to the increment of base when combined with the sulfate or phosphate radicle. This is true in spite of the fact that the concentrations for beginning precipitation are different in the case of sodium and potassium phosphates, a difference which is too great to be accidental.

The evidence which Brönsted (13) has obtained from a study of the solubility of inorganic compounds in salt solutions has many points in common with the data presented in this paper on complex protein substances. This investigator finds:

"1. The activity coefficient of an ion may be determined by two factors, one of which is due to the salting-out effect of the salt solution serving as solvent and the other to electrical interaction between the said ion and the ions of the solvent.

2. Ions are uniformly influenced by ions of their own sign. Their activity coefficients depend, therefore, only upon the action of ions of opposite sign and the salting-out effect of the solvent.

3. The salting-out effect of a salt solution can be represented as a product of the salting-out effects of the separate ions."

Brönsted's data were obtained by comparing the solubility of various inorganic compounds in the presence of salt solutions of equal total ion concentration, whereas our data relate to the concentrations of salt which have equal salting-out ability. Under such conditions we have found that sodium and potassium salts of the same structure are approximately equally effective, and the ratios of the concentrations required as sulfate to those required as chloride are the same for these cations. The latter fact apparently holds for the beginning concentration for lithium chloride, but on the basis of the increment of salt lithium as a chloride is relatively twice as effective as when present as a sulfate. The relation between sodium and potassium phosphates is not that which exists for the sulfates with regard to the beginning

concentration, but it holds for the increment of salt. These facts agree in general with Brönsted's findings that the ratio of the salting-out capacities of two cations is the same for any anion and that a similar relation holds for two anions with a common cation.

An explanation will not be attempted at present of the salting-out of protein. The relatively limited knowledge with regard to the nature of concentrated solutions and the assumptions which must be made in order to account for certain effects in the salting-out of inorganic compounds do not admit of more than speculation with regard to the salting-out of proteins. We are inclined to the acceptance of the idea that the phenomenon is related to processes of solubility and salting-out which obtain with inorganic salts. This is essentially an acceptance of the fundamental conception first expressed by Spiro (8) and confirmed by Hardy (18), that salting-out could best be explained as a separation into phases. Spiro introduced the idea that there was a qualitative specific factor, solution intensity, which should be taken into consideration. Loeb (19) has recently presented evidence to show that the conditions which are responsible for the stability of proteins in solution and the precipitation of proteins by high concentrations of salts represent the forces of secondary valency, developed by Langmuir, responsible for the stability of crystalloids in general. On the basis of Loeb's distinction between colloidal properties and crystalloidal properties of protein solutions, our data are more in agreement with his evidence on the crystalloidal properties of proteins. McBain and Salmon (20) assume the possibility of soap existing in either the crystalloidal or the colloidal state.

The significance of the threshold concentration, *i.e.* the concentration of salt required to bring a protein solution to the point where the addition of an increment of salt will cause precipitation, and of the increment of salt must be left for further study. If the relation between the degree of hydration of the various proteins of blood and the quantity of salt required to precipitate a protein fraction holds, as suggested by Chick (21), then the quantity of water held by successive protein fractions is less for each fraction by an equal amount; her data do not show this relation,

there is some question as to the accuracy of the calculations based on her determinations, which does not, however, detract from the general value of the results.³

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STUDIES IN INORGANIC METABOLISM.

I. THE INFLUENCE OF COD LIVER OIL UPON CALCIUM AND PHOSPHORUS METABOLISM.

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To discuss here the importance of further information on mineral metabolism is, I believe, superfluous. This Journal has recently contained several interesting papers concerning this subject from Hart and coworkers, Sherman and collaborators, Meigs and associates, McCollum and colleagues, Forbes and coworkers, and others. Some of these dealt with the influence of cod liver oil on mineral metabolism. Notwithstanding this fact the results mentioned in this paper contribute, in my opinion, additional information.

For these experiments the use of small animals is preferable. With them it is not too expensive to give pure salts and other pure substances (lactose, etc.), so that the different elements and organic substances can be given in all desirable proportions and the amounts varied to such an extent as to procure decisive or striking results.

One of the purposes of these experiments was to ascertain the way in which one can improve the mineral metabolism of milch cows. I, therefore, chose rabbits for the experimental animals, as the composition of their diet does not differ much from that of the ruminants.

The further purpose of these experiments was to investigate the effect of cod liver oil on different physiological features; *i.e.*, whether this oil influences the mineral metabolism by altering the fecal or the urinary excretion; and whether it causes the mineral saving with diets poor in calcium as well as when the intake of calcium surpasses the usual output. Also, I intended to study the relationship between calcium metabolism

and reproduction. The experiments with cod liver oil were followed by experiments on the influence of indigestible material (crude fiber) in the food on the Ca and P metabolism. They were suggested by the results of the cod liver oil experiments, which proved that the weight of the feces decreased when the oil was administered. It was thought probable that the opposite effect would occur, a higher fecal calcium output resulting when the bulk of feces was increased by giving a diet with a high amount of crude fiber.

EXPERIMENTAL.

The investigations were carried out on two adult rabbits, No. 1, a male, weighing about 3,400 gm., and No. 2, a female, weighing about 2,000 gm. They were confined separately in iron metabolism cages and kept out of direct sunlight. The diet consisted of a food mixture, the composition of which is given below, and whole wheat kernels, in the ratio 3:1. Later it contained butter (in the first experiments 5 gm. daily, afterwards 3 gm.) and 15 gm. of cabbage (from March 4 to 14, 20 gm.).

Each day 60 gm. of the mixture, 20 gm. of wheat, and the butter were mixed with 125 cc. of hot distilled water and given to each rabbit. The following day the part of the ration that was not consumed was weighed. The cabbage was given separately from the rest of the food.

From May 26 the amount of water was increased to 175 cc. (*i.e.* in Period II of the second experiment on Rabbit 1).

The rabbits were completely adapted to the routine before the experiment was begun.

Composition of the Food Mixture.

Casein.....	15.0	Previously dissolved in NaOH, precipitated with lactic acid, washed several times, and dried.
Oat straw.....	3.0	Previously boiled with sulfuric acid and NaOH, washed, and dried.
K ₂ HPO ₄	1.0	
NaCl.....	0.6	
MgSO ₄ +7H ₂ O.....	0.5	
Citric acid.....	0.4	
Dextrin.....	52.9	
Lactose.....	26.5	
Total.....	99.9	

0.1 of a mixture of ferric citrate, KI, and MnSO₄.

The diet seemed to be acid-forming as we found the pH of the urine in several samples to be 5.4 to 5.0. The wheat and casein must have been the cause for this. After treatment the casein contained only a small amount of calcium, *i.e.* between 0.2 and 0.3 per cent, and 1.53 per cent H_3PO_4 (determined after destruction).

Urine and feces were collected daily, placed together in a solution of thymol in chloroform and, with some exceptions, kept for a period of 7 days. Analyses were made upon these composite samples (except the estimations of creatinine and of total nitrogen, which were made in the fresh urine collected one morning). The feces were dried and analyzed in an air-dried condition.

The Ca determinations were made by McCrudden's method as well as by Lyman's nephelometric method, after ashing the feces and foods and after destruction of the urine with nitric acid.

The phosphorus was determined in the air-dried feces and foods, after destruction with sulfuric acid, potassium sulfate, and copper sulfate, by Bell and Doisy's method, modified by Briggs.¹ Also, the phosphorus determinations were controlled by a different method; *i.e.*, nephelometrically by Bloor's method, revised by Kleinmann.

The administration of cod liver oil was easy with one of the rabbits (No. 2), as it took the oil spread on cabbage leaves. With Rabbit 1 it was necessary to pour the oil into the mouth of the animal with the aid of a syringe. The oil was previously shaken up in water.

1 gm. of cod liver oil was given daily. Two experiments on Rabbit 1 consisted of three periods, each of which lasted at least 14 days. The period of cod liver oil administration (Period II) being preceded (Period I) and followed (Period III) by a period in which the diet was the same except that 1 gm. of butter replaced 1 gm. of cod liver oil.

During the first experiment (from Mar. 4 until Apr. 24) the diet was poor in calcium, during the second (from May 2 until June 12) a supplement of calcium acetate was given, containing 40 mg. of Ca daily, which made the diet rich in calcium. The calcium acetate was spread over the cabbage in order that it might be entirely consumed.

The experiment on Rabbit 2 was practically the same as the first experiment on Rabbit 1 and was made simultaneously. The diet, therefore, was poor in calcium.

It must be taken into account that the collecting of urine and feces of Period II, the experimental period proper, began on the 1st day of cod liver oil administration and ended on the last day that this oil was consumed. It would have been more correct not to consider the first few days as belonging to the period.

¹ Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 15.

TABLE I.
Experiment with Cod Liver Oil on Rabbit I. Diet Poor in Calcium.

	Period I. Without cod liver oil.					Period II. With cod liver oil.				Period III. Without cod liver oil.			
	Mar. 4 to 10	Mar. 11 to 17	Mar. 18 to 22	Mar. 23 to 29	Mar. 4 to 22	Mar. 30 to Apr. 9	Mar. 23 to Apr. 9	Apr. 10 to 17	Apr. 18 to 24	Apr. 18 to 24	Apr. 10 to 24	Apr. 18 to 24	Apr. 10 to 24
Date.....	7	7	5	19	1	7	11	18	1	8	7	15	1
No. of days.....													
Calcium.													
Intake, mg.....	180.0	169.7	132.3	482	25.37	199.6	296.6	496.2	27.57	182.0	175.3	357.3	23.82
Output in urine, mg.....	24.4	33.4	37.9	95.7	5.0	28.0	29.6	57.6	3.2	62.4	79.6	142.0	9.47
Output in feces, mg.....	179.7	200.5	240.9	621.1	32.7	109.5	104.0	213.5	11.86	192.5	207.1	399.6	26.64
Total output, mg.....	204.1	233.9	278.8	716.8	37.7	137.5	133.6	271.1	15.06	254.9	286.7	541.6	36.1
Balance, mg.....	-24.1	-64.2	-146.5	-234.8	-12.33	+62.1	+163.0	+225.1	+12.5	-72.9	-111.4	-184.3	-12.28
Loss in per cent													
Gain of intake, mg....	-13.4	-38.0	-110.7	-48.7	-48.7	+31.1	+55.0	+45.4	+45.4	-40	-63.5	-51.6	-51.6
In 100 cc. urine,													
mg.....	7.3	14.5	26.5			19.6	12.75			20.8	47.8		
In 100 gm. air- dried feces, mg.	378	606.0	644.0			450.5	292.0			515.0	595.0		
Volume of urine, cc.....	334	230	143	707	37.2	143	232	375	20.8	299	166.5	465.5	31.0
Weight of air- dried feces, gm.	31.1	33.1	31.5	95.7	5.037	24.3	35.6	59.9	3.33	37.0	34.8	71.8	4.787

Phosphoric acid.

Intake, <i>mg</i>	3,640	3,830	2,632	10,102	531	3,388	5,262	8,650	481	3,507	3,052	6,559	438
Output in urine, <i>mg</i>	1,456	1,518	787	3,761	198	954	1,230	2,184	121.3	1,071	1,395	2,466	164.4
Output in feces, <i>mg</i>	976	1,041	999	3,016	159	542	953	1,495	83.0	1,191	1,202	2,303	159.6
Total output, <i>mg</i>	2,432	2,559	1,786	6,777	357 +174	1,496	2,183	3,679	204.3 +276.7	2,262	2,597	4,859	324 +114
Balance, <i>mg</i>													
In 100 cc. urine, <i>mg</i>	436	600	550			667	530			357	838		
In 100 gm. air- dried feces, <i>mg</i> .	3,138	3,145	3,171			2,232	2,678			3,218	3,455		

TABLE II.
Experiment with Cod Liver Oil on Rabbit 2. Diet Poor in Calcium.

	Period I. Without cod liver oil.					Period II. With cod liver oil.					Period III. Without cod liver oil.		
	Mar. 4 to 10	Mar. 11 to 17	Mar. 18 to 22	Mar. 23 to 29	Mar. 4 to 22	Mar. 23 to 29	Mar. 30 to Apr. 5	Mar. 23 to Apr. 9	Apr. 10 to 17	Apr. 18 to 24	Apr. 10 to 24		
	7	7	5	7	7	7	11	18	1	8	7	15	1
No. of days.....													
Calcium.													
Intake, <i>mg.</i>	205.2	181.3	135.5	522	27.47	199.6	305.9	505.5	28.08	154.4	147.2	301.6	20.1
Output in urine, <i>mg.</i>	52.5	24.0	18	94.5	5.0	27.5	38.0	65.5	3.64	40.2	63.8	104.0	7.0
Output in feces, <i>mg.</i>	497.5	455.4	379	1,331.9	70.0	182.0	157.3	339.3	18.85	75.9	173.3	249.2	16.6
Total output, <i>mg.</i>	550.0	479.4	397	1,426.4	75.0	209.5	195.3	404.8	22.5	116.1	237.1	353.2	23.6
Balance, <i>mg.</i>	-344.8	-298.1	-261.5	-904.4	-47.6	-9.9	+110.6	+100.7	+5.6	+38.3	-89.9	-51.6	-3.5
Loss in per cent of Gain													
Intake, <i>mg.</i>	-168	-164	-193	-173.3	-173.3	-5.0	+36.2	+19.7	+19.7	+24.8	-61.0	-17.1	-17.1
In 100 cc. urine, <i>mg.</i>	17.5	8.0	12.5			9.3	9.5			9.9	21.1		
In 100 gm. air-dried feces, <i>mg.</i>	906	848	925			755.0	277.5			395.4	1,030.5		
Volume of urine, cc.....	229	300	144	743	39.1	296	400	696	38.7	405	302.5	707.5	47.2
Weight of air-dried feces, <i>gm.</i>	54.9	53.7	41	149.6	7.875	24.1	56.7	80.8	4.49	19.2	16.8	36.0	2.4

Phosphoric acid.

Intake, <i>mg.</i>	4,252	4,105	2,740	11,097	584	3,388	5,500	8,888	494	2,611	2,156	4,767	318
Output in urine, <i>mg.</i>	951	900	700	2,551	134	1,302	2,980	4,342	241	1,549	1,134	2,683	179
Output in feces, <i>mg.</i>													
Total output, <i>mg.</i> ...	2,662	2,607	2,033	7,302	384	933	1,355	2,288	127	417	848	1,265	84
Balance, <i>mg.</i>	3,613	3,507	2,733	9,853	518	2,295	4,335	6,630	368	1,966	1,982	3,948	263
In 100 cc. urine, <i>mg.</i>	318	300	486		+66		720		+126				+55
In 100 gm. air-dried feces, <i>mg.</i>	4,850	4,855	4,960			3,870	2,390			2,170	5,050		

Phosphoric acid.

Intake, <i>mg</i>	4,252	4,105	2,740	11,097	584	3,388	5,500	8,888	494	2,611	2,156	4,707	318
Output in urine, <i>mg</i>	951	900	700	2,551	134	1,302	2,980	4,342	241	1,549	1,134	2,683	179
Output in feces, <i>mg</i>	2,662	2,607	2,033	7,302	384	933	1,355	2,288	127	417	848	1,265	84
Total output, <i>mg</i> ...	3,613	3,507	2,733	9,853	518	2,205	4,335	6,630	368	1,966	1,982	3,948	263
Balance, <i>mg</i>					+66				+126				+55
In 100 cc. urine, <i>mg</i>	318	300	486			460	720			385	375		
In 100 gm. air-dried feces, <i>mg</i>	4,850	4,855	4,960			3,870	2,390			2,170	5,050		

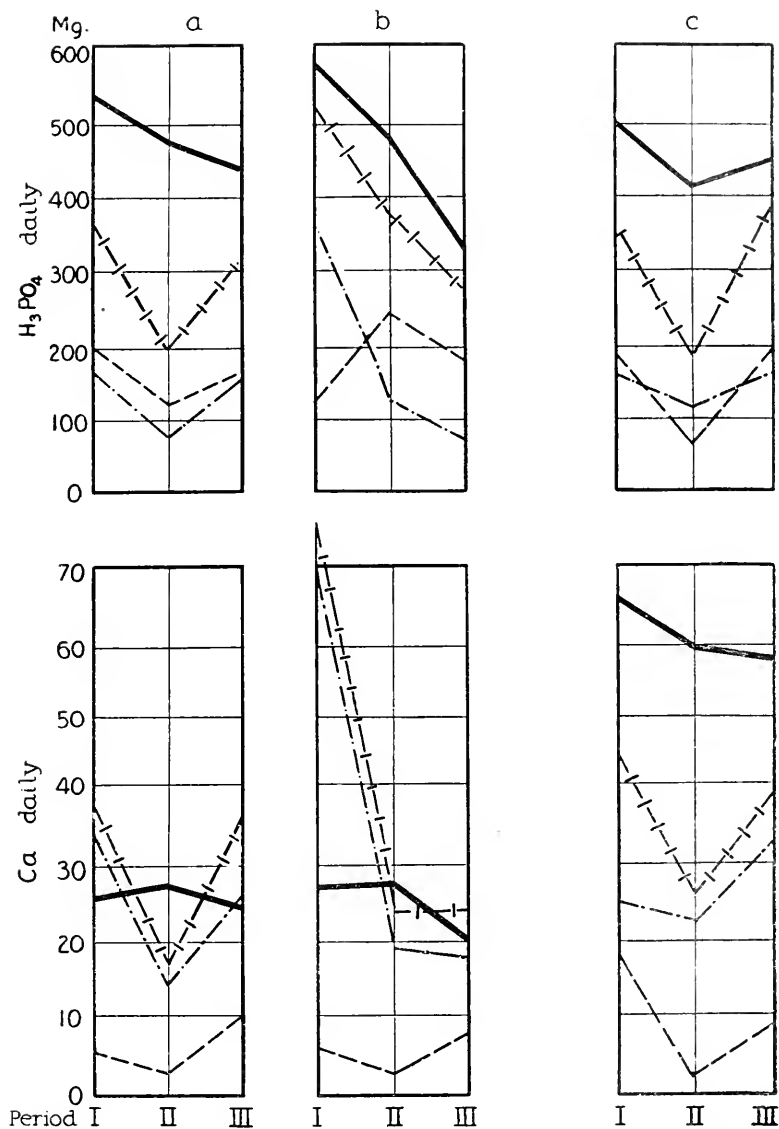


CHART 1, *a* to *c*. Period II represents the period of administration of cod liver oil. (*a*) Diet poor in Ca; Rabbit 1. (*b*) Diet poor in Ca; Rabbit 2. (*c*) Diet rich in Ca; Rabbit 1. —Intake; —|—Total output; --- Output in urine; --- Output in feces.

DISCUSSION.

Tables I, II, and III and Chart 1 *a*, *b*, and *c* show the details concerning the Ca metabolism of three experiments with cod liver oil.

The total Ca output of Rabbit 2 in the initial period (Period I) was two and one-half to three times higher than the intake (Table II and Chart 1 *b*). The Ca balance of Rabbit 1 was also markedly negative in this period of the first experiment; the ratio of intake to output being 1:1.5. With both rabbits the Ca balance was positive during Period II, but became negative soon afterwards, *i.e.* when the administration of cod liver oil was stopped; even in the first week of Period III the balance was negative with Rabbit 1.

The great losses of calcium prove that the rabbit easily loses the calcium of the bones. Rabbit 2 lost in some periods about 50 mg. daily; *i.e.*, about twice the amount of calcium that was in the blood. If a rabbit contains about 1.5 per cent of Ca, there were 30 gm. of Ca in Rabbit 2. The daily loss, therefore, comes to 0.1 per cent of the body calcium. During the initial period of 19 days it was 904 mg.; *i.e.*, 3 per cent. These experiments give once again positive proof of the great influence of cod liver oil when the diet is poor in calcium.

The result of the second experiment on Rabbit 1 demonstrates the same to be true for a diet rich in calcium. In this experiment, as is shown in Table III and Chart 1 *c*, the daily gain of calcium during the administration of cod liver oil (Period II) was 35.15 mg.; during Periods I and III it was 21.2 and 20.4 mg.

Considering the fecal and urinary output separately we can state that, during the experiments with diets poor in calcium, the amount of Ca in the urine was very low. The urine contained only 6.6 to 13.2 per cent of the total output. A considerable reduction of it was excluded. In the experiment with a rich calcium diet the urinary Ca output forms a much greater part of the total Ca output; *i.e.*, in Period I it was 42.3 per cent. It made a sharp fall in Period II; the daily Ca output in the urine being in Period I 18.62 mg. and in Period II (administration of cod liver oil) 2.16 mg. (Table III and Chart 1 *c*).

Nearly the whole economy of calcium produced by the administration of cod liver oil in this experiment is due to the decreased output in the urine, the difference between Periods I

and II in total calcium output being 18.9 mg., in urinary output 16.46, and, therefore, in fecal excretion only 2.45 mg. daily. These experiments give the interesting result that cod liver oil reduces the renal calcium excretion when the diet contains more calcium than the total output, and that in contrast to this it forces the organism to economize calcium by diminishing the fecal calcium where the balance is highly negative. A saving of calcium in the feces was obtained in both possible ways; *i.e.*, the production of a smaller amount of feces and a smaller content of Ca in the feces. In Period I of the first experiment Rabbit 1 produced daily 5.04 gm. of air-dried feces, during the experimental period proper 3.33, and afterwards again 4.8 gm. (Table I and Chart 1 a). For Rabbit 2 these figures were 7.88, 4.49, and 2.4 gm. Concerning the last figure it must be noted that during the last 2 weeks (Period III) Rabbit 2 took much less food than in the preceding periods. During the periods of cod liver oil administration the daily food intake was not much smaller than in the initial periods. For Rabbit 1 (first experiment) the ratio was 48.5:43; for Rabbit 2 it was 53:45 (leaving the daily portion of cabbage out of consideration).

In the experiment rich in Ca on Rabbit 1 there was not the same decrease in the production of feces. It was in the three periods: 4.66, 4.28, and 5.62. The relative food intakes (cabbage excluded) were 45.5, 38, and 41.5.

The second way in which the fecal Ca output decreased, was the smaller Ca percentage. With Rabbit 1 (first experiment) it fell from about 0.6 to about 0.3 and afterwards increased again to about 0.6 per cent. A similar decrease was observed with Rabbit 2. In Period I the percentage was 0.9, in Period II it sank to 0.28 per cent of Ca, and afterwards rose again considerably.

In the experiment with a rich Ca diet the effect of the cod liver oil on the Ca percentage in the feces exists in a smaller degree. It was in the three periods: 0.55, 0.45, and 0.62 per cent.

During this experiment the greatest saving of calcium was obtained by the decrease of the percentage in the urine and also by the smaller volume of the urine which was voided daily. As the feces in the two experiments with a poor Ca diet in several periods contained much more calcium than the food, it is obvious

that it is impossible, by analyzing the feces, to tell whether the calcium of a certain food is available or not; a great part of the fecal calcium not being directly originated by the food. Moreover, the decrease in the calcium of the feces caused by the administration of cod liver oil has nothing to do with the availability of the calcium in one or more of the constituents of the diet. From this it is evident that the digestibility of the calcium of a certain food can be highly influenced by other constituents of the diet. The same holds good for phosphorus. Nevertheless, it may be possible to compare the availability of calcium or phosphorus in a certain food or salt by analyzing the feces, provided there is no change in the other constituents of the food. The interesting result that with a diet deficient in calcium the feces contain under certain circumstances much more calcium than the food (for instance, the fecal output of Rabbit 2 contained daily 70 mg. and the intake 27 mg. of calcium) proves that the excretion of calcium is connected with the production of feces or in general with the functions of the intestines. Even when the organism loses nearly three times as much calcium as it receives, the percentage of calcium in the feces remains the same (see Rabbit 2, March 18 to 22; Table II). We shall see later that the amount of calcium in the feces cannot be explained by the excretion of Ca soap.

The intestines, therefore, do not merely excrete the calcium, but use it for their functions. It seems to be easier for the organism to decrease the Ca content of the urine, so long as the Ca percentage of the urine does not reach the minimum level, than to decrease that of the feces.

The Ca requirement of the intestines is decreased apparently considerably by the administration of cod liver oil and perhaps by that of other products containing the fat-soluble A.

When comparing the calcium excretion in the two experiments on Rabbit 1 we find that there was good retention of the calcium from calcium acetate. The diet was in all respects the same except that calcium acetate, furnishing 40 mg. of calcium daily, was added. The total Ca output in the second experiment surpasses that of the first only by 6.3, 9.9, and 2.5 mg. of Ca in the three periods. The Ca retention (difference between intake and output) was in the three periods of the second experiment, respectively 21.2, 35.2, and 20.4 per cent of the calcium present in the food.

In three samples of feces of the first experiment (poor Ca diet) on Rabbit 1 the amount of total bases was determined by the method of Fiske.² The total bases of the feces of the second collection of Period I were equivalent to 1,035 cc., those of the second collection of Period II (the experimental period proper) to 835 cc., and those of the second collection of Period III with 1,220 cc. of 0.1 N alkali. If we subtract the calcium, we find for the other bases, respectively, 732, 689, and 922.5 cc. These figures show that the excretion of the other bases also diminishes in the feces during the administration of cod liver oil.

The same conclusion may be drawn from the determinations on raw ash of the feces of Rabbit 1 in the experiment with a rich Ca diet. The two feces collections of Period I gave a total ash of 7.5 and 7.4 per cent; those of Period III 7.3 and 7.2 per cent; whereas during the administration of cod liver oil the figures were 6.7 and 6.6 per cent.

From some of the samples of feces the percentage of soaps was determined in order to examine whether these constituents could have been of influence on the results.

We made an ether extraction of the dry feces without a previous treatment, and one after digestion with alcoholic hydrochloric acid. The difference was considered to be fatty acid originally present as soap.

The percentage of fat (fatty acids) plus fatty acids combined as soap was in most samples between 2.5 and 4; the highest was 5.27; the lowest 1.2 per cent. The percentage of fatty acids present as soap was usually less than 1, the highest was 1.65.

The percentage of calcium in the feces present as soap was in most samples about 10; calculating all soap as Ca soap, the highest figure was 16 per cent. Neither the administration of cod liver oil nor the addition of calcium acetate had an appreciable influence on this percentage. It is evident, therefore, that the formation of soap has had no influence of importance on the fecal Ca output in these experiments.

From these analyses it may be concluded that the resorption of fat from the food was satisfactory. From the fat intake (about 30 gm.) between 1 and 2 gm. were usually found in the feces.

² Fiske, C. H., *J. Biol. Chem.*, 1922, li, 55.

Concerning *phosphorus metabolism* Tables I, II, and III and Chart 1 *a, b,* and *c* show that cod liver oil economizes phosphorus as well as calcium. It has, therefore, about the same value for the improvement of the P metabolism as for that of the calcium. In both experiments on Rabbit 1 the intake was larger than the total output, yet the decrease in output in Period II, both in urine and feces, was evident. It is of interest to notice that the P output was not influenced by the amount of calcium in the food. There was no difference of importance between the P excretion in urine and feces in the corresponding periods of the experiment with a poor Ca and of that with a rich Ca diet except those in Period III, which were, however, caused by a smaller consumption of food in the first experiment. The results concerning the P metabolism of the experiment on Rabbit 2 in general agree with those on Rabbit 1 although there is an increase in the renal phosphorus in Period II which cannot be explained. During Period III the food intake decreased largely and as a consequence the P output did not increase; yet there is a sharp turn in the curve of the fecal P excretion.

Contrary to calcium the P output in the urine is often higher than that in the feces. This may be caused by the relatively high amount of P in the food. The percentage of phosphorus in the feces decreased considerably through the administration of cod liver oil in all three experiments, so there was to a certain extent a confluence between the Ca and P output in the feces. Nevertheless, there were differences of importance as the percentage of calcium of the feces decreased in proportion more than that of phosphorus, so that the ratio, which was in other periods 1:5 or 6, became 1 calcium to about 9 H_3PO_4 . Perhaps cod liver oil has more influence on the economy of calcium than of phosphorus. The economy by decreasing the urinary phosphorus seems to be due more to the diminishing volume of urine than to the lower percentage of phosphorus.

As is seen from the tables and charts a highly negative calcium balance coincides during Periods I and III of both experiments on a poor calcium diet with a positive P balance. If we accept the fact that the greater part of the excreted calcium is derived from the calcium phosphate of the bones, we have to believe that phosphorus can be stored in some other tissue or tissues; probably as lecithin in the liver and perhaps also in the bone

marrow. However, we have to consider the possibility that all, or nearly all, the calcium is derived from the calcium carbonate of the bones if the phosphate and carbonate do not occur in the bones in a more complex combination. The ratio Ca:P in the urine was during these periods usually greater during experiments with a positive Ca balance.

During these experiments the amount of creatinine plus creatine in several samples of urine and of creatinine alone was determined colorimetrically by the picrate method. For this purpose fresh samples were used (not those collected weekly). One striking feature of the result of these analyses may be mentioned. In all three experiments the percentage of creatinine plus creatine (and also that of creatinine alone) increased in the first days of the administration of cod liver oil. With Rabbit 2 it was about 300 mg. per 100 cc. of urine in Period I; the first day of Period II it was about 500. In the first days after this period it fell again considerably. In the first experiment on Rabbit 1 the urine contained in the first period about 400 mg.; during the second between 900 and 700; afterwards it decreased. In the second experiment on Rabbit 1 (addition of calcium) the creatinine content of the urine per 100 cc. was during Period II about double that before the administration of cod liver oil; after Period II it fell lower than in Period I.

It must be taken into account, however, that the amount of urine in Period II of all three experiments was less than in the other periods (see Tables I, II, and III). The difference was large, especially in the second experiment on Rabbit 1 (rich Ca diet). As the decrease in the volume of the urine which was voided, was in some cases smaller than the increase in percentage of creatinine, it seems provisionally that the minimal endogenous level of protein metabolism is increased by cod liver oil. I intend to reexamine this point.

The decrease in urine excretion through the administration of cod liver oil may be considered as a striking result. Even the increase of daily water intake in the middle of the second week of Period II of the experiment with a Ca-rich diet of 50 cc. did not raise the volume of urine.

One of the conclusions of these experiments may be that we must reckon with the possibility that the diseases (keratomalacia, etc.), ascribed to diets deficient in fat-soluble A, are caused by

lack of inorganic elements, the deficiency of this unknown dietary factor causing the deficit in retention of calcium, phosphorus, etc.

SUMMARY AND CONCLUSIONS.

These experiments on rabbits show that as the result of administration of cod liver oil the Ca and P loss is decreased when the calcium balance is negative, and that when it is positive the retention is increased. In the first case it is principally the fecal Ca output, in the second it is the urinary output, that is decreased.

Cod liver oil diminishes the production of feces if the Ca output greatly surpasses the intake, that of urine if the diet is rich in Ca. There is reason for the supposition that the fat-soluble A (or the specific vitamin of cod liver oil) prevents the consequences of diets deficient in this dietary factor through the influence it has on the mineral metabolism.

Where the feces sometimes contain much more Ca than the food, it is evident that the availability of the calcium in the food or in one or more constituents of the food cannot under all circumstances be determined by analyzing the feces. This is impossible because of the great influence that cod liver oil and probably also other substances have on the Ca retention and also on the amount of Ca output in the feces.

The total bases in the feces decrease when the content of calcium diminishes.

The total output of P is independent of the amount of Ca present in the food. A highly negative calcium balance can coincide with a positive phosphorus balance, even when the total Ca output is about three times higher than the intake.

The ratio of Ca:P in the feces was about the same in all samples except in those collected during the cod liver oil periods.

Rabbits can excrete amounts of Ca that are about three times the Ca present in the food and still give feces with the same percentage of Ca as in periods of positive Ca balance. The organism, therefore, excretes Ca from the bones more easily than it produces feces with low Ca content. This points to a physiological action of importance of Ca for the functions of the intestines, especially in connection with feces formation. It may also be considered as a reason to accept a great mobility of the inorganic constituents of the bones.

STUDIES IN INORGANIC METABOLISM.

II. THE INFLUENCE OF CRUDE FIBER AND OF PROTEIN UPON CALCIUM AND PHOSPHORUS METABOLISM.

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On the adult rabbit, No. 1, used for the experiments with cod liver oil, the influence of different amounts of indigestible crude fiber in the diet on the Ca and P metabolism was examined. The reason for these experiments was the result mentioned in the preceding paper, that the administration of cod liver oil caused a smaller production of feces and thereby decreased the fecal calcium output. It was therefore of interest to determine whether an increase of the amount of roughage¹ promotes a greater loss of calcium and phosphorus. In connection with the practical bearings of these experiments; for instance, for the feeding of milch cows, I chose crude fiber as roughage. Experiments with sand or agar-agar would also have been of interest.

With one of the two experiments was combined an examination of the influence of an increase of protein intake on the Ca and P metabolism.

EXPERIMENTAL.

As in the experiments described in the foregoing paper, the diet consisted of the food mixture and wheat in the ratio 3:1, butter 4 gm. daily, and green food. During the summer months it was not possible to get cabbage; therefore, mangels, carrots, and carrot tops were substituted during a part of the first roughage experiment. Mangels (15 gm. daily) were given from May 30 until July 8. From that date till Aug. 1 the animal received daily 2 gm. of carrot tops and 10 gm. of carrots; thereafter 15 gm. of carrots.

¹ I use the term "roughage" in this paper to indicate the indigestible constituents of the food.

TABLE I.
First Experiment.

Period.....	I	II	III	IV	V	VI	VII
Date.....	May 30 to June 12	June 13 to 19	June 20 to July 3	July 4 to 17	July 18 to 31	Aug. 1 to 7	Aug. 8 to 21
Length of period, <i>days</i>	14	7	14	14	14	7	11
Roughage.....	3 per cent oat straw.	Gradual in- crease.	10 per cent oat straw; 5 per cent sawdust.	As in Period III.	As in Period III.	Gradual decrease.	3 per cent sawdust.
Supplements.....	40 mg. Ca daily.	As in Pe- riod I.	As in Pe- riod I.	10 per cent wheat glu- ten; 0.18 gm. cystine daily; Ca as in Period I.	Cystine as in Period IV; Ca as in Pe- riod I; no wheat glu- ten.	15 mg. Ca daily.	15 mg. Ca daily.

TABLE II.
Second Experiment.

Period.....	I	II	III	IV	V
Date.....	Sept. 8 to 21	Sept. 22 to 28	Sept. 29 to Oct. 12	Oct. 13 to 19	Oct. 20 to Nov. 2
Length of period, <i>days</i>	14	7	14	7	14
Roughage.....	3 per cent oat straw.	Transition to 15 per cent oat straw.	15 per cent oat straw.	Transition to 3 per cent oat straw.	3 per cent oat straw.
Supplements.....	12.7 mg. Ca as acetate daily.	6.35 mg. Ca daily.	No suppli- ment.	6.35 mg. Ca daily.	As in Period I.

In the second roughage experiment 15 gm. of cabbage were again used.

In the roughage periods the food mixture contained 15 per cent roughage—oat straw or sawdust previously boiled with dilute sulfuric acid and solution of sodium hydroxide—which was 12 per cent more than usual. As the diet was composed of 3 parts of food mixture to 1 part of wheat, the diet contained (if the crude fiber of the green food and of the wheat is not taken into account) about 11.25 per cent of roughage during the roughage periods. In the periods of high roughage or high protein the amounts of dextrin and lactose were decreased to the same extent (in the ratio 2:1).

During the first roughage experiment the 15 per cent of roughage consisted of 10 per cent oat straw and 5 per cent sawdust; during the second experiment 15 per cent oat straw was given.

Another difference between the two experiments was that, during the first, a supplement of calcium acetate, containing 40 mg. of Ca daily, was procured, while, during the last two periods (Periods VI and VII) this supplement was decreased to 15 mg. of Ca.

As both the prepared oat straw and sawdust contained more calcium than the other constituents of the food mixture the diet was richer in Ca during Periods III, IV, and V than in the other periods of the first experiment. This objection was eliminated in the second experiment by giving such supplements of calcium acetate that the percentage of calcium was the same in all periods. The methods of analysis, the way in which the food was given, the shading from sunlight, the collecting of feces and urine, etc., were the same as in the experiments with cod liver oil. The periods of the experiments are shown in Tables I and II.

In reference to the supplement of cystine, about 0.180 gm. daily during Periods IV and V of the first roughage experiment, it may be mentioned that the rabbit became bald during the foregoing weeks; *i.e.*, on the back (behind the ears) a space of about 10×10 cm. was without hair. As casein is very poor in cystine I tried the influence of this amino-acid. The result was marvelous. In about 14 to 20 days all the bald spots were covered with thick fur.

In Period IV of the first roughage experiment the food mixture contained 15 and 10 per cent of wheat gluten, whereas in all preceding and following periods the amount of protein in the mixture was 15 per cent of casein.

The animal was weighed every 3rd day. The weight varied little. It was usually between 3,500 and 3,600 gm.

DISCUSSION.

Tables III to VI and Charts 1 and 2 show the influence of a high content of crude fiber in the diet on the Ca output.

The results of the first experiment are more convincing than those of the second. During the latter the diet was poorer in

TABLE III—*First Roughage Experiment on*

	Period I. 3 per cent roughage in the mixture.			Period II.	Period III. 15 per cent roughage in the mixture.		
Date.....	May 30 to June 5	June 6 to 12	May 30 to June 12 daily.	June 13 to 19	June 20 to 26	June 27 to July 3	June 20 to July 3 daily.
Cal-							
Intake, <i>mg.</i>	408.6	418.0	59.0	489.9	518.9	559.1	77.0
Output in urine, <i>mg.</i>	40.5	74.9	8.25	257.5	248.0	195.6	31.7
“ “ feces, <i>mg.</i>	225.8	199.3	30.4	239.7	503.7	735.5	88.5
Total output, <i>mg.</i>	266.3	274.2	38.6	497.2	751.7	931.1	120.2
Balance, <i>mg.</i>	+142.3	+143.8	+20.4	-7.3	-232.8	-372.0	-43.2
In 100 cc. urine, <i>mg.</i>	10.0	14.8		55.6	41	26.8	
“ 100 gm. air-dried feces, <i>mg.</i>	485.0	621		655	690	793	
Volume of urine, <i>cc.</i>	405.0	506.0	65.0	463	605	730	95.3
Weight of air-dried feces, <i>gm.</i>	46.55	32.1	5.62	36.6	73.0	92.75	11.87
Phosphor-							
Intake, <i>mg.</i>	3.084	3.284	455	3.188	3.644	4.564	585
Output in urine, <i>mg.</i>	1.093	1.725	202	1.755	2.033	2.263	307.0
“ “ feces, <i>mg.</i>	1.289	1.027	165	1.014	1.774	2.122	278.3
Total output, <i>mg.</i>	2.382	2.752	367	2.769	3.807	4.385	585.1
Balance, <i>mg.</i>	+702	+532	+88	+419	-163	+179	+1.14
In 100 cc. urine, <i>mg.</i>	270.0	341.0		379	336	310	
“ 100 gm. air-dried feces, <i>mg.</i>	2,770	3,200		2,772	2,430	2,288	

calcium than during the former, so that the negative balance was greater. This brought about a large fall in the urine calcium. At the same time that the large amount of roughage caused a high fecal Ca output, the calcium in the urine became so much less that it nearly compensated the increase of fecal output.

The ratio of the fecal calcium in Period I to that in the experimental periods proper was for the first experiment about 100:250; and for the second 100:150. It may be observed that the calcium

of the roughage itself cannot have influenced the increase in fecal calcium, as the 12 per cent of roughage contained in the first experiment about 19 mg. daily, in the second 9.4 mg. of Ca. The increase in fecal calcium during the roughage periods is much larger.

Rabbit 1. Diet Not Poor in Calcium.

Period IV. 15 per cent roughage and high protein in the mixture.			Period V. 15 per cent roughage in the mixture.			Period VI.	Period VII. 3 per cent roughage in the mixture.		
July 4 to 10	July 11 to 17	July 4 to 17 daily.	July 18 to 24	July 25 to 31	July 18 to 31 daily.	Aug. 1 to 7	Aug. 8 to 14	Aug. 15 to 21	Aug. 8 to 21 daily.
585.7	673.9	90.0	524.6	522.7	74.8	245.0	326.45	322.1	46.3
213.0	307.5	37.2	234.8	192.0	30.5	120.0	85.1	129.4	15.3
402.0	458.0	61.4	614.9	355.4	69.3	154.5	107.7	67.7	12.5
615	765.5	98.6	849.7	547.4	99.8	274.5	192.8	197.1	27.8
-29.3	-81.6	-8.6	-325.1	-24.7	-25.0	-29.5	+133.65	+125.0	+18.5
37.7	62.0		43.4	29.0		29.0	27.8	42.3	
600.0	657.0		726.0	570.5		344	369	384.5	
565.0	476.0	74.3	541.0	664.0	86.1	413.0	306.0	306.0	43.7
67.0	69.7	9.8	84.7	62.3	10.5	44.9	29.2	17.6	3.34

acid.

4,486	4,470	639	4,206	4,446	618	4,083	3,739	3,683	530.1
1,808	1,828	259.7	2,164	1,912	291.1	1,751	1,677	1,610	235.0
1,481	1,538	215.6	1,793	1,121	208.1	965	647	408	75.3
3,289	3,366	475.3	3,957	3,033	499.3	2,716	2,324	2,018	310.1
+1,197	+1,104	+164.4	+249	+1,413	+118.7	+1,367	+1,415	+1,665	+220
320	384		400	288		424	548	526	
2,210	2,207		2,117	1,800		2,150	2,216	2,320	

Especially in the second experiment, the results show that only a small part of the intestinal excretion of Ca is in some circumstances directly derived from the food; *i.e.*, in Period III of the second experiment the daily intake was 35.0 mg., the fecal calcium 66.8 mg.

The daily losses of calcium are during some periods still larger than in the experiments with cod liver oil; they were about 70 mg. or 0.14 per cent of the body calcium. Because of the bulk

Balance, <i>mg</i>	+222	+456	+48.7	+53	-606	+170	-30.6	+299	+30	-68	-2.2
In 100 cc. urine, <i>mg</i>	223.0	374		397	445	381		336	356	430	
" 100 gm. air-dried feces, <i>mg</i>	3,152	2,350		2,100	1,457	1,440		1,595	1,995	1,360	

Nitrogen.

N in urine, <i>per cent</i>	1.215	0.99			1.325	1.1		1.39	1.27	2.14	
N in air-dried feces, <i>per cent</i>	3.22				2.365	2.05		1.64	2.1	2.814	
Output in urine, <i>gm</i>	6.33	3.18	0.843	5.43	6.47	5.7	0.87	5.42	5.23	7.32	0.896
" " feces, <i>gm</i>	0.94	5.47	0.116	1.16	1.2	1.18	0.17	1.13	0.6	0.72	0.094
Total N output.....	7.27	0.69	0.96	6.59	7.67	6.88	1.04	6.55	5.83	8.04	0.99

of air-dried feces the total fecal Ca was high in the roughage periods.

The figures in Table VII give the daily excretions in grams.

The weight of the 12 per cent (15.3 mg.) extra roughage daily present in the food was for the first experiment about 6.6, for the second 4.7 gm. These figures do not differ much from the differences in fecal excretion. The calcium percentage of the feces was in the first experiment higher during the roughage

TABLE V.
First Experiment.

	Period I.	Period III.	Period V.	Period VII.
Roughage, <i>per cent.</i>	3	15	15	3
Ca intake daily, <i>mg.</i>	59	77	74.8	46.3
Total Ca output daily, <i>mg.</i>	38.6	120.2	99.8	27.8
Gain (+); loss (-) daily, <i>mg.</i>	+20.4	-43.2	-25	+18.5
Fecal Ca output daily, <i>mg.</i>	30.4	88.5	69.3	12.5

TABLE VI.
Second Experiment.

	Period I.	Period III.	Period V.
Roughage, <i>per cent.</i>	3	15	3
Ca intake daily, <i>mg.</i>	33.6	35	36.4
Total Ca output daily, <i>mg.</i>	93.4	104.4	29.6
Gain (+); loss (-) daily, <i>mg.</i>	-59.8	-69.4	+6.8
Fecal Ca output daily, <i>mg.</i>	44.1	66.8	21.1

periods than in Period I, notwithstanding the fact that in Period I the balance was positive. Only at the end of the last roughage period was the percentage decreased, perhaps because of the negative balance of the preceding weeks. In Period I the feces contained 0.55 per cent. during the two roughage periods with normal protein content, 0.7 per cent of Ca. During the roughage period with high protein the percentage was 0.63 per cent.

In the second experiment (with the poor Ca diet) there was a decrease of the percentage of calcium in the feces; it fell from 1.2 to 1. It has already been seen that the percentage of Ca in the urine decreased greatly in the second experiment; *i.e.*, from 64

to 15.5. Just as in the experiments with cod liver oil the calcium of the urine falls with negative balances more easily than that of

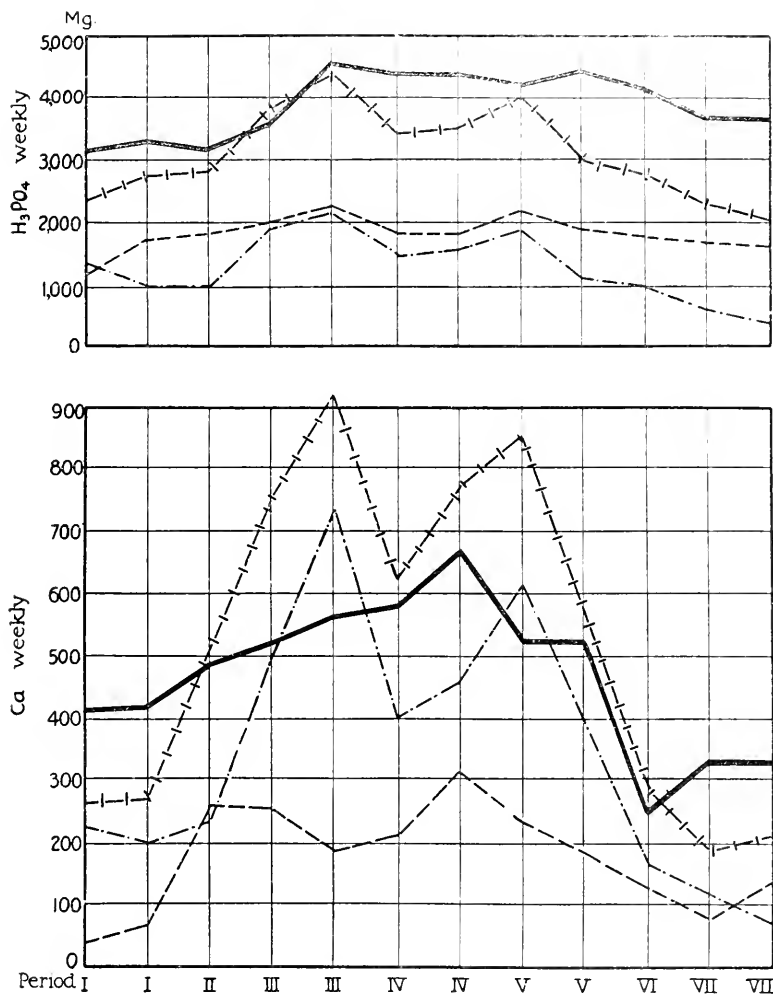


CHART I. First roughage experiment, Rabbit I. — Intake; - - Total output; - - - Output in urine; - · - Output in feces.

the feces. One might expect that milch cows, kept on a ration with much crude fiber, will not retain much calcium, by diminish-

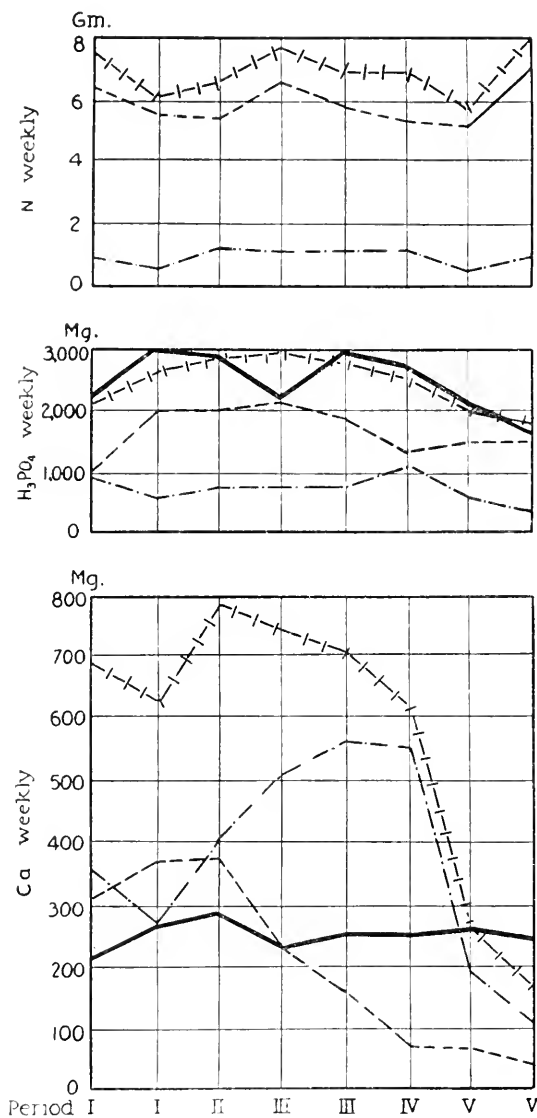


CHART 2. Second roughage experiment, Rabbit 1. — Intake; -|- Total output; --- Output in urine; -.- Output in feces.

ing the urinary calcium percentage, since their urine is as a rule poor in calcium.

Concerning the urine in the first experiment (with a diet not so poor in calcium), it may be observed that the urinary calcium excretion was much higher during the roughage periods than before; about four times higher. This was principally due to the higher Ca concentration, the volume of the urine being, during the roughage periods, only about 50 per cent greater than in Period I. Therefore, we see here the contrary to what happened in the second experiment where the decrease in urinary calcium nearly compensated the increase of fecal calcium.

We now have to examine the effect of the *high protein intake* on the calcium metabolism. Table III and Chart 1 show the calcium economy it gave. In Period III the output surpasses the intake daily by 43.2 mg.; in the roughage protein period (Period IV)

TABLE VII.

Experiment No.	Period I.	Period III.	Period V.	Period VII.
1	5.62	11.9	10.5	3.35
2	3.62	7.72	3.85	

by only 8 mg., and in the following period the 1st week again gave a large loss; *i.e.*, 46 mg. daily. These figures prove that a high protein diet favorably influences mineral retention. On the phosphorus output there was a similar influence.

Examining separately the fecal and urinary Ca output we find during the high protein period a large decrease in fecal calcium; it fell from 88.5 to 61.4 mg. daily, and rose in the following week again to 88.0 mg. Notwithstanding the smaller volume of urine voided during the high protein period, the urinary Ca output was little higher than in the two other roughage periods.

From this experiment, which must be repeated, it appears that there is a relation between the intake of nitrogen and the metabolism of Ca and P. The reverse is not the case, at least not in the same degree. The nitrogen output in the second roughage experiment was very little influenced by the high content of roughage. Especially does this hold good for the fecal nitrogen.

It may be deduced from the determination of total ash in the feces of Periods I, II, and III of the first roughage experiment

that there is a decrease in the percentage content of the other bases in the feces when the amount of roughage in the food is raised. The percentages of total ash in the 2 weeks of Period I were 7.3 and 7.3, in Period II (transition period) 6.5, and in the 1st week of Period III 5.3 per cent. The same conclusion may be drawn from the determinations of total bases by the method of Fiske in the feces of the 2nd week of Period I and of the 2nd week of Period III (both of the first roughage experiment). The percentage of Ca being in these two samples 0.62 and 0.79 per cent; the total bases being equivalent to 851 and 510 cc. of 0.1 N alkali. The feces of the 1st week of Period V (high protein) with 0.6 per cent Ca contained an amount of total bases equivalent to 762 cc. of 0.1 N alkali. It may be that protein stimulates the metabolism of some minerals.

It may be advisable to call attention to the fact that in Period V (first roughage experiment) the total Ca output was only very little higher than in the period with high protein in the food. The figures were 1,397 and 1,380. The influence of the high protein diet (decrease in calcium output), therefore, is best demonstrated by the great fall in protein from Period III to IV. Probably a negative Ca balance lasting some weeks, will in general prevent an increase of the losses.

Concerning the phosphorus metabolism I have already made some remarks. The P output does not quite follow that of the Ca (see Tables III and IV and Charts 1 and 2). The roughage increases the fecal and also the urinary P output only to an insignificant extent in both experiments. The percentage of phosphorus in the feces diminished during the roughage periods and afterwards rose again; the decrease was most evident in the second experiment (poor Ca diet). As the Ca percentage of the feces increased or decreased only a little, it is clear that there was an inverse tendency or at least no confluence concerning Ca and P. Therefore, there is not always a definite relation between these two, even when the amount of Ca and P in the food remains unchanged. The same was found during the administration of cod liver oil.

Phosphorus does not seem to play the same rôle in the intestines as does calcium.

It is worth while to examine the influence of an increased protein level on the P metabolism. Chart 1 shows clearly the diminished output during this period. This decrease is principally due to the smaller urine and feces production.

It would be of importance to learn whether the diminished production of urine through the influence of protein or of cod liver oil continues over long periods.

Concerning the practical bearings, especially with respect to the mineral metabolism of milch cows, it may be mentioned that the content of the crude fiber of rations of cows can differ largely depending on the amount of straw, hay, etc., and also on the percentage of crude fiber of the roughage used. There can easily be a difference of 1 kilo of indigestible crude fiber in the daily rations of a cow. In reference to this I point to the fact that the crude fiber of different hay samples can differ more than 14 per cent. If a cow gives 5 kilos of air-dried feces on a ration poor in crude fiber, it will produce 6 kilos or even more with other foods. This makes a difference of 20 per cent or more in feces production, as well as in the fecal Ca output.

One of the problems that I wished to examine was the influence of negative Ca balances on reproduction, when there are Ca losses over a long period. We twice tried to mate the male rabbit, No. 1; both times without result. Of course, a greater number of matings would have been necessary to get decisive results.

After having been used about a whole year for the experiments described in these two papers and also for others Rabbit 1 died on November 16 from liver cirrhosis, as shown by the autopsy. In the Anatomical Institute of my colleague Dr. G. Krediet, the testicles have been found to show microscopical anatomical abnormalities which he describes as follows.

One of the testes was observed to be normal, macroscopically; the other was too small. The first contains an interstitium with interstitial cells and seminiferous tubules, which are normal.

Concerning the spermatogenesis it may be remarked, that there are only a few spermatozoa which have tails. Spermatoocytes and spermatids are vacuolized and degenerating in great numbers. Spermatogones and Sertoli's cells are normal.

It is possible that the cirrhosis of this rabbit was due to the diet poor in calcium. No other foods than those mentioned in these papers were given during a whole year.²

SUMMARY AND CONCLUSIONS.

An increase of indigestible crude fiber in the diet causes a higher fecal output of Ca. When the diet is poor in Ca this increase may be partly compensated by the decrease in urinary calcium output.

Even when the Ca balance is highly negative there was a large percentage of Ca in the feces during the periods with a relatively high roughage in the food. As in the experiments with cod liver oil the urinary calcium falls sooner than the fecal calcium.

A high protein diet decreases the fecal Ca output. An increase of roughage in the diet barely influences the nitrogen output. The same is the case with the P output. Therefore Ca and P do not always concur and probably have not the same physiological action in the intestines. As in the experiments with cod liver oil a highly negative Ca balance coincides with a positive P balance, even when—as in some periods of the second roughage experiment—the total Ca output is more than three times higher than the intake. The production of feces and urine was diminished by protein just as it was by cod liver oil.

A diet poor in calcium, given over a long period, may have been the cause of loss of reproductive power in one animal, and of microscopic anatomical abnormalities of the testicles.

A ration with a large amount of indigestible crude fiber increases in milk cows the risk of a negative calcium balance. This is one more disadvantage of hay and other roughage of bad quality.

In conclusion I desire to express my sincere appreciation of the assistance of Miss J. E. Van der Zande, and Messrs. H. Wooghoudt and W. Gieteling in carrying out the great number of analyses; also of Mr. G. J. Naber for his attendance of the animals.

² Wells published in 1903 a paper in which he mentions the observation that repeated injections of Witte's peptone (chiefly proteoses) into rabbits led to the production of marked cirrhosis of the liver. In his book (Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, 576) he says: "subsequent observations, however, have shown that repeated injections of almost any foreign protein material will cause a similar cirrhosis in rabbits, which animals, indeed, often spontaneously show this condition when apparently otherwise normal."

2nd line from the bottom of the page, for "W. Gieteling" read "H. Gieteling."

STUDIES OF THE THYROID APPARATUS.

XVII. THE EFFECT OF THYROPARATHYROIDECTOMY AND PARATHYROIDECTOMY AT 100 DAYS OF AGE ON THE Ca, Mg, AND P CONTENT OF THE ASH OF THE HUMERUS AND FEMUR OF MALE AND FEMALE ALBINO RATS.

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The preceding paper (1) of this series dealt with the gross anatomical and chemical differentiation of the humerus and femur of male and female albino rats during the growth period from 100 to 150 days of age, and the effects of thyroparathyroidectomy and parathyroidectomy thereon. The gross anatomical differentiation was determined from measurements of weight and length; the gross chemical, from determinations of the water, organic matter, and ash content of the bones. This paper reports a similar study of the ash of these bones and is based on determinations of their calcium, magnesium, and phosphorus content.

The source of the material and the general plan of the experiment have been described in an earlier communication (2). The preparation of the bones for analysis and the procedure up to the point where the ash was obtained are to be found in the preceding paper (1). The further technique was as follows: The ash was dissolved in the silica crucible with 1 to 2 cc. of concentrated HCl, transferred to a 50 or 100 cc. volumetric flask, according to weight, and made up to volume with distilled water. Aliquots were taken in duplicate; calcium was determined by the method of Kramer and Tisdall (3), phosphorus by the method of Bell and Doisy (4), and magnesium by the modified method of Hammett and Adams (5). Certain of the earlier analyses were made according to the method of Hammett and

TABLE I.
Statistical Data of the Calcium, Magnesium, and Phosphorus Content of the Ash of the Humerus and Femur of Various Groups of Male Rats.

Humerus.					Femur.				
Ash.	Calcium.	Magnesium.	Phosphorus.		Ash.	Calcium.	Magnesium.	Phosphorus.	
Reference controls. 100 days old. 41 rats.									
gm.	mg.	per cent	mg.	per cent	gm.	mg.	per cent	mg.	per cent
0.0703	26.30	37.40	0.600	0.85	12.91	18.34	0.1457	51.45	37.32
Mean.....									
Standard deviation.....	0.0138	5.19	0.18	0.133	0.05	2.65	0.33	0.0358	13.61
Probable error of mean.....	0.0028	1.06	0.04	0.027	0.01	0.54	0.07	0.0073	2.77
Coefficient of variability.....	19.63	19.74	0.47	22.20	6.13	20.53	1.79	21.37	25.00
Controls. 150 days old. 41 rats.									
0.1031	38	73.37	52	0.899	0.86	18.87	18.25	0.2140	80.38
Mean.....									
Standard deviation.....	0.0153	5.88	0.70	0.199	0.09	3.09	0.58	0.0342	13.22
Probable error of mean.....	0.0031	1.20	0.14	0.041	0.02	0.63	0.12	0.0069	2.69
Coefficient of variability.....	14.81	15.18	1.85	22.15	10.58	16.39	3.20	15.98	16.15
Thyroparathyroidectomized rats. 150 days old. 12 rats.									
0.0811	30	54.37	55	0.770	0.93	15.02	18.51	0.1708	63.98
Mean.....									
Standard deviation.....	0.0169	6.81	0.62	0.268	0.18	3.18	0.49	0.0403	15.59
Probable error of mean.....	0.0033	1.33	0.12	0.052	0.03	0.62	0.10	0.0078	3.03
Coefficient of variability.....	20.84	22.29	1.65	34.79	19.08	21.15	2.65	23.37	21.37
Parathyroidectomized rats. 150 days old. 14 rats.									
0.0900	33	62.37	49	0.921	1.03	17.05	18.95	0.1941	72.57
Mean.....									
Standard deviation.....	0.0181	6.59	0.77	0.181	0.09	3.33	0.39	0.0474	17.22
Probable error of mean.....	0.0033	1.19	0.14	0.033	0.02	0.60	0.07	0.0085	3.10
Coefficient of variability.....	20.11	19.61	2.05	19.63	8.69	19.54	2.03	24.42	23.72

TABLE II.

Statistical Data of the Calcium, Magnesium, and Phosphorus Content of the Ash of the Humerus and Femur of Various Groups of Female Rats.

	Humerus.				Femur.									
	Ash.	Calcium.	Magnesium.	Phosphorus.	Ash.	Calcium.	Magnesium.	Phosphorus.						
Reference controls. 100 days old. 10 rats.														
	gm.	mg.	per cent	mg.	per cent	gm.	mg.	per cent						
Mean.....	0.0729	27.57	37.83	0.605	0.84	13.47	18.47	0.1539	57.99	37.66	1.291	0.81	28.56	18.57
Standard deviation.....	0.0087	4.20	0.31	0.051	0.06	1.58	0.15	0.0241	9.37	0.37	0.178	0.05	4.41	0.20
Probable error of mean.....	0.0019	0.90	0.07	0.011	0.01	0.34	0.03	0.0051	2.00	0.08	0.038	0.01	0.95	0.04
Coefficient of variability.....	11.93	15.25	0.83	8.48	6.60	11.75	0.81	15.66	16.16	0.98	13.82	5.62	15.53	1.07
Controls. 150 days old. 11 rats.														
Mean.....	0.0962	36.36	37.80	0.826	0.85	17.60	18.29	0.2066	75.85	37.76	1.703	0.85	37.01	18.45
Standard deviation.....	0.0166	5.84	0.60	0.245	0.16	3.22	0.78	0.0418	15.92	0.48	0.429	0.10	8.36	0.50
Probable error of mean.....	0.0034	1.19	0.12	0.050	0.03	0.66	0.16	0.0085	3.24	0.10	0.087	0.02	1.70	0.10
Coefficient of variability.....	17.26	16.06	1.59	29.69	18.94	18.31	4.27	20.84	20.99	1.26	25.20	12.25	22.59	4.92
Thyroparathyroidectomized rats. 150 days old. 13 rats.														
Mean.....	0.0686*	25.56	37.28	0.634	0.92	12.60	18.47	0.1519	56.55	37.22	1.358	0.89	28.28	18.56
Standard deviation.....	0.0103	3.76	0.55	0.137	0.14	1.85	0.62	0.0941	11.28	0.40	0.330	0.10	6.18	0.71
Probable error of mean.....	0.0020	0.73	0.11	0.027	0.03	0.36	0.12	0.0176	2.11	0.07	0.062	0.02	1.46	0.13
Coefficient of variability.....	15.00	14.69	1.48	21.62	14.86	14.67	3.38	61.95	19.95	1.07	24.28	10.89	21.85	3.81
Parathyroidectomized rats. 150 days old. 15 rats.														
Mean.....	0.0763	28.23	36.98	0.789	1.03	14.40	18.84	0.1626	60.14	36.92	1.655	1.01	30.88	18.93
Standard deviation.....	0.0151	5.82	0.44	0.181	0.09	2.99	0.49	0.0349	13.43	0.48	0.397	0.07	7.08	0.38
Probable error of mean.....	0.0026	1.01	0.08	0.032	0.02	0.52	0.09	0.0061	2.34	0.08	0.069	0.01	1.23	0.07
Coefficient of variability.....	19.79	20.61	1.20	23.28	8.75	20.77	2.63	21.46	22.33	1.31	21.00	7.00	22.91	1.98

* One set of analyses was lost; therefore, the data on the ash weight were calculated from the 12 bones.

Adams (6) as first described and may well have given too high values. But since all the bones of any given set were analyzed by but one method, intergroup comparison is not vitiated. Only the statistical data of the results obtained are tabulated here. The individual records are on file at The Wistar Institute and are available for study.

In Table I are found the statistical data of the ash and the absolute and percentage values of calcium, magnesium, and phosphorus content of the humerus and femur of the male reference control (7), control, thyroparathyroidectomized, and parathyroidectomized groups of rats. Table II gives the same data obtained from the females. The reference controls were 100 days of age, the other groups 150 days of age at the time the bones were taken for analysis. It should be noted that the percentage values represent the mean percentage of calcium, magnesium, and phosphorus, not the percentage content of the average ash in these constituents. They, therefore, differ slightly from the values obtained by direct calculations from the means of the absolute amounts.

Normal Bone.

At 100 days of age the percentage value of calcium, magnesium, and phosphorus of the humerus ash is practically the same as that of the femur, notwithstanding the markedly heavier ash and greater absolute amount of the three constituents in the latter. There is, however, a slight but valid superiority in the percentage of phosphorus exhibited by the femur. Since this is present in both sexes, and is shown in all the groups, though not to a statistically valid degree in all (the consistency of the positive deviation for the individual observations is 66 per cent), it is undoubtedly significant. This consistent superiority of the femur in organic matter percentage (1) is the most plausible explanation.

At 100 days of age no significant sex difference is exhibited in the percentage of magnesium and phosphorus. The percentage of calcium in the ash of both bones, however, is greater in the females than in the males. This sex difference is an accompaniment of the more advanced state of ossification of the bones in the females as shown by their higher percentage of ash (1).

At 150 days of age the identity of the humerus and femur ash in calcium and magnesium percentage, the superiority of the femur in percentage of phosphorus, and the higher percentage of calcium in the bones of the females are shown, as at 100 days of age, and are similarly interpretable.

The sex differences in absolute amounts of ash, and of calcium, magnesium, and phosphorus of the ash at both ages are too small to be individually valid, yet the consistency of the direction of deviation shows that the bones of the females at 100 days of age have absolutely greater amounts of ash, calcium, magnesium,

TABLE III.

Percentage Increments of Calcium, Magnesium, and Phosphorus of the Various Groups of Rats During the Growth Period from 100 to 150 Days.

Element.	Controls.				Thyroparathyroidectomized rats.				Parathyroidectomized rats.			
	Males.		Females.		Males.		Females.		Males.		Females.	
	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.	Humerus.	Femur.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Calcium.....	47.3	47.6	31.9	30.8	16.1	17.5	-7.3	-2.5	27.8	38.8	2.4	3.7
Magnesium.....	49.8	39.4	36.5	31.9	28.3	26.9	4.8	5.2	53.5	53.3	30.4	28.2
Phosphorus.....	46.1	47.7	30.8	29.6	16.3	17.7	-6.4	-1.0	32.1	36.5	7.0	8.1

and phosphorus than do those of the males, and that at 150 days the conditions are reversed. The correctness of this supposition is supported by the fact that the rate of growth of the humerus and femur in the male rats during the period of observation is greater than that of the females (1) in weight, length, water, organic matter, ash, calcium, magnesium, and phosphorus.

The study of growth is the study of a process. The chief focus of these investigations is on the changes incident to growth and the effects of thyroid (thyroparathyroid) and parathyroid lack on these changes.

Although the percentage of ash content of both bones of both sexes increases because the rate of growth in weight by ash depo-

sition is greater than the rate of growth in weight of the bone as a whole (1) and the absolute ash content increases as a result of the deposition of calcium, magnesium, and phosphorus, no valid changes in the percentage content of the ash in these constituents obtain.

Certain drifts appear, such as a tendency towards an increased calcium percentage and a decreased phosphorus percentage, but the extent of the change is too small to be anything but an indication of a possible tendency. It is therefore evident that the nature of the ash, in as far as its calcium, magnesium, and phosphorus make-up is concerned, laid down in these bones during normal growth from 100 to 150 days of age, is practically identical with that already present, and that there is no definitely detectable differentiation in ash composition occurring over this interval.

The extent of the increase in the ash constituents determined is of interest in that it brings out several points of importance. In Table III there are given the percentage increments of calcium, magnesium, and phosphorus of the ash for the period of observation.

It is evident that there is an almost complete uniformity in percentage increase of the three constituents in the individual bone; that the percentage increase is practically the same in both bones, and that it is uniformly greater in the males than in the females.

Before going on to a specific consideration of the changes induced by the experimental procedures I wish to call attention to certain general implications afforded by the data as a whole.

The uniformity of the percentage increase and composition of the two bones is exhibited by the experimental groups as well as by the controls. This indicates that the chemical processes concerned with ossification (the deposition of the calcium, magnesium, and phosphorus compounds characteristic of osseous tissue) are identical for serially homologous bones, notwithstanding sex differences in intensity, or qualitative alterations of activity due to thyroid (thyroparathyroid) or parathyroid deficiency.

Certain general trends in group variability are of significance. The variability in percentage composition is consistently much

less than the variability in absolute amounts. This demonstrates that regardless of the amount of ash present or being formed in the process of ossification the bone tends to maintain that adequate balance between the amounts of calcium, magnesium, and phosphorus of the ash which yields a uniform percentage composition of the ash with respect to these three constituents. Although experimental procedure may cause a shift in the processes and distribution, the tendency to maintain the percentage composition characteristic for the changed state of the organism remains.

The order of increasing variability in percentage values is Ca, P, Mg. The differences are sufficiently consistent and marked to be valid. The difference between the group variability in calcium and phosphorus percentage is much less than that between calcium or phosphorus and magnesium. It is probable, however, that the high variability in magnesium percentage is methodological. This opinion is supported by the fact that the group variability in absolute amounts of magnesium is generally higher than that of calcium or phosphorus. The slightly greater variability in percentage phosphorus as compared with calcium may be technical or due to organic matter combination variability.

Bones of the Thyroparathyroidectomized Rats.

As in the control group, the ash content and the absolute amounts of calcium, magnesium, and phosphorus of the femur of this group were greater than those of the humerus in both sexes, while the percentage composition of the two bones was practically the same, although a tendency to superiority in phosphorus percentage was exhibited by the male femur.

The thyroparathyroidectomized rats differed from the controls in that sex differences in absolute amounts of the ash and its constituents were present. The bones of the females had less ash, calcium, magnesium, and phosphorus than did those of the males. As in the control groups, so in this, there were no significant sex differences in magnesium or phosphorus percentage of the ash. But the calcium percentage tended to be higher in the males than in the females; a reversal of the condition in the controls. This is explained by the greater sensitivity of the

female organism to thyroid deficiency, as described in previous papers.

Comparing now the state of the bones of the thyroparathyroidectomized rats with those of their controls of the same age it is seen that both bones of both sexes of the operated groups have absolutely less ash, calcium, magnesium, and phosphorus. These bones were also lighter, shorter, and had less water and organic matter (1). In the males the percentage of calcium of both bones of the thyroparathyroidectomized rats did not differ to a statistically valid degree from that of their controls. There was a greater phosphorus percentage in the humerus which was reflected in the femur to a slight extent, and which can be associated with the greater percentage of organic matter (1) or with the increase in percentage of magnesium or both. The female thyroparathyroidectomized rats had a definitely lower calcium percentage than their controls, but they showed no valid changes in percentage of phosphorus although the drift to a higher value as exhibited in the males was evident. As in the males, so in the females, the percentage of magnesium was higher in the thyroparathyroidectomized rats than in the controls. The general trend, therefore, of the two sexes was the same.

An analytical comparison of the terminal state of the bones of the control and test groups fails to give a valid basis for an interpretation of the effect of the experimental procedure on the process of ash formation. An analysis of a terminal state by itself is no index of the process which has led to that state. The lack of appreciation of this principle has led to many erroneous conclusions. It is only by comparing initial and terminal states, with intermediary interpolations when possible, that an adequate picture is obtained.

A study of Table I shows that the absolute amount of calcium, magnesium, and phosphorus of the ash of both bones of the male thyroparathyroidectomized rats at 150 days of age was greater than that of their reference controls 100 days old, and less than that of their controls of the same age. From Table III it is evident that the percentage increase in absolute amount of these three constituents was markedly less than that of the controls for the same period, although the differences of both bones from the controls were qualitatively the same and quantitatively of

the same order of magnitude. In the thyroparathyroidectomized rats the percentage increase in calcium, magnesium, and phosphorus was not uniform in degree as was the case in the controls. The percentages of calcium and phosphorus increments were the same, but definitely lower than that of magnesium.

It is thus evident that the lack of thyroid (thyroparathyroid) secretion has not only retarded ash formation (1) and the deposition of the salts of calcium, magnesium, and phosphorus, but it has also caused a change in the nature of the ash. This differential effect of thyroid (thyroparathyroid) deficiency on the relative rate of deposition of the ash constituents naturally resulted in an alteration or differentiation of the percentage composition of the ash. The result of the differentiation was the production of an ash having approximately the same calcium percentage as the normal, a slightly higher magnesium percentage, and a tendency towards a higher percentage of phosphorus. It is to be remembered that no definitely detectable differentiation of ash composition normally occurs during the growth of the bones from 100 to 150 days of age.

Since these observations represent the effects of chronic thyroparathyroid deficiency they are not strictly comparable with observations made on thyroparathyroidectomized animals moribund from acute parathyroid tetany or kept alive by special therapeutic measures. As will be shown presently, however, it is probable that the slight shift in ash composition is due to the parathyroid and not to the thyroid deficiency.

Marie Parhon (8) has recently reported that chronic thyroid deficiency in sheep results in a low calcium content of the blood. The studies of Hastings and Murray (9), Gross and Underhill (10), and others show that during the period of acute parathyroid tetany following thyroparathyroidectomy the blood calcium is low. Although all these effects, with the possible exception of those obtained by Parhon, may be attributed to the parathyroid deficiency, it is probable that thyroid deficiency as well as parathyroid deficiency produces a lowering of the calcium-retaining ability of the organism.

This belief is founded on the fact reported in the preceding paper (1) that while ossification of the humerus and femur in the male thyroparathyroidectomized rats is both absolutely and

relatively inhibited so that these bones not only contain less ash but a smaller percentage of ash, in the male parathyroidectomized rats the percentage of ash of the bones is no different from that of the normal controls of the same age.

In other words, since parathyroid deficiency in the male rat does not prevent that degree of ossification of the bones consistent with their growth and size, while a combined thyroid and parathyroid deficiency has this effect, it is probable that the diminution of ossification ability is largely a specific effect of the lack of thyroid function.

It is necessary to differentiate between ossification as the general process concerned in the deposition of the inorganic salts characteristic of bone, and calcification as the process in ossification concerned with the deposition of calcium salts. Ossification can be considered as being factored by at least two processes: (1) the ability to lay down the inorganic salts, and (2) the ability to lay them down in their correct proportions. The one is quantitative, the other qualitative. The one is a matter of growth, the other a matter of differentiation. It is possible for growth (ossification) to be retarded and differentiation (calcification) to be not greatly disturbed. Such has occurred in the bones of these male thyroparathyroidectomized rats for Table I shows that the percentage of calcium is normal.

Therefore, thyroid deficiency in the males, even though it causes a retardation of absolute growth of the two bones by increments in water, organic matter, and ash, even though it causes a decrease in the extent of ossification, and even though it probably causes a decrease in the ability of the organism to retain calcium, does not cause any loss of calcium from these bones or prevent the formation of an ash of normal calcium content.

Turning to the male parathyroidectomized rats it is seen from Table I that the absolute amount of calcium, magnesium, and phosphorus of the ash of both bones at 150 days of age was greater than that of their reference controls 100 days old. During growth, increments of these constituents were made. These increments were greater than those laid down in the bones of thyroparathyroidectomized rats. Comparing the data of parathyroidectomized rats with those of the controls of the same age

it is found that the absolute amount of calcium and phosphorus was less in the tests. This indicates some degree of retardation of growth by deposition of these constituents. On the other hand, the parathyroidectomized rats tended to have a higher amount of magnesium than their controls. It is obvious that disturbance of ash composition has been produced. The direction of this disturbance and the extent of the effect of parathyroid lack are brought out in Table III.

The percentage increase in calcium and phosphorus was diminished to a considerable extent, but not to anywhere near the degree that it was in the thyroparathyroidectomized rats. This, of course, is due to the fact that the toxemia of parathyroid deficiency did not affect adversely the growth of the bones in weight, length, water, organic matter, and ash increments as much as did the thyroid (thyroparathyroid) deficiency (1).

The percentage increase in magnesium was greater in the parathyroidectomized rats than in the controls; again, a quantitatively different response than that elicited by thyroparathyroidectomy. The response of the three constituents with regard to distribution was the same for both groups. Moreover, the differences of both bones from those of the controls were qualitatively the same and quantitatively of practically the same order of magnitude, just as was observed in the thyroparathyroidectomized rats.

The lack of parathyroid secretion not only retarded ash formation (1) to a slight extent and the deposition of calcium, magnesium, and phosphorus, but it caused a change in the nature of the ash. This disturbance is reflected in the percentage composition of the ash given in Table I. The result of the disturbance was the production of a bone having practically the same percentage of calcium as that of the normal, but a much higher percentage of magnesium and phosphorus. This shift in percentage composition of the ash was in the same direction, but over twice as far from the controls as occurred in the thyroparathyroidectomized rats. It is the expression of the true parathyroid deficiency effect. The high phosphorus content of the bones is in line with the observations of Greenwald (11) and Juschtschenko (12) that a phosphorus retention occurs after thyroparathyroidectomy and parathyroidectomy as shown by the decreased excretion and increased concentration in the blood.

Since the shift in the percentage composition of the ash of the thyroparathyroidectomized rats was in the same direction it is probable that the slight disturbance in the latter was also an expression of the lack of parathyroid secretion. A tentative explanation for the damping down of the effects of parathyroidectomy when the thyroid has been simultaneously removed has been given in another place (13).

The literature contains no record of a disturbance in magnesium metabolism following parathyroidectomy such as is described here. That it is a real phenomenon is shown by its uniform occurrence in both bones of both sexes.

These analytical results show definitely that the lack of parathyroid function does not cause an absolute or a relative loss of calcium from the bones of male rats. They show that the ability of the bone to incorporate into its ash the normal proportion of calcium is undisturbed, even though an absolute loss of calcium from the organism may have occurred, as indicated by the experiments of MacCallum and Voegtlin (14) even though a low blood calcium may be present as shown by the studies of these investigators. Trendelenburg and Goebel (15), and even the histological studies of Erdheim (16), Morel (17), Canal (18), and others tend to demonstrate a deficient ossification, which my analyses have established as a fact (1).

This is significant of an inherent stability of the chemical process of calcification and removes it from a direct dependence upon parathyroid function. It is, to my mind, a further obstruction to the theory of MacCallum.

The reactions in the females were somewhat different in certain respects.

Table II shows that after thyroparathyroidectomy the ash of both bones failed to increase in absolute amount. In fact, the humerus of the female thyroparathyroidectomized rats at 150 days of age contained less ash than did the humerus of their reference controls 100 days old. While the femur showed the same drift the difference was not large enough to be valid. This resulted in a decrease in the percentage of ash of both bones and is indicative of an inhibition of ossification similar to that observed in the males.

Since there was no increase in ash it was to be expected that the ash constituents would fail to show an increase in absolute amounts, and this is seen to be the case in both bones in as far as calcium and phosphorus are concerned. In fact, there was a general tendency for these constituents to be less in the ash of thyroparathyroidectomized rats than in the ash of the reference controls, 50 days younger. In the humerus the differences were almost sufficiently great to be statistically valid. The magnesium, on the other hand, showed no decrease, but a tendency towards a higher value. Although many of these differences are small and cannot be considered valid when taken individually, the fact that the drift is uniform for both bones justifies the belief that the direction of change induced by thyroparathyroidectomy in the females is towards a bone (1), having absolutely and relatively less ash, less absolute amounts of calcium and phosphorus in the ash, and more magnesium.

The trend of these changes is shown better by the data in Table III. They show that qualitatively the differences of both bones from the controls were the same, and that although the inhibitions in the increase of ash by the individual constituents were greater in the females than in the males, the relative distribution of the response was the same in both sexes.

That is to say, the percentage change in calcium, magnesium, and phosphorus was not uniform as was the case in the controls. The calcium and phosphorus were more adversely affected than was the magnesium. The response of the calcium and phosphorus was of the same order of magnitude in both bones, although probably slightly less marked in the femur.

It is thus evident that the lack of thyroid (thyroparathyroid) secretion has initiated a change in the nature of the ash of both bones of the females. The result of this disturbance, as seen from the data on percentage composition of the bones in Table II, was the production of an ash having a definitely lower percentage of calcium than the controls of both ages, a slightly higher magnesium percentage, and a tendency towards a higher percentage of phosphorus than was present in the controls of the same age.

The course of differentiation in magnesium and phosphorus was quite the same as in the ash of the bones of the male thyro-

parathyroidectomized rats and is similarly interpretable. The absolute and relative decrease in calcium content of the bones of the females is evidence of decalcification, a phenomenon which did not occur in the males. An explanation of this will be given after a description of the results of parathyroid deficiency.

From Table II it is evident that the absolute amount of calcium, magnesium, and phosphorus of the ash of both bones of the parathyroidectomized females was consistently greater than that of their reference controls at 100 days of age, though only to a statistically valid degree in the case of the phosphorus in the femur and of the magnesium of both bones. Nevertheless, the consistency of the drift is sufficient to justify the belief that during growth, increments of ash and calcium, magnesium, and phosphorus of the ash were made. These increments were consistently greater than those laid down in the thyroparathyroidectomized rats. Comparing the data of the parathyroidectomized rats with those of their controls of the same age it is seen that the absolute amounts of calcium and phosphorus were less in the tests than in the controls, while the magnesium was greater. Thus it is seen that the direction of change was the same in the females as in the males and is similarly interpretable.

The direction and extent of the disturbance is brought out in Table III.

The percentage increase of all constituents of the parathyroidectomized females was of the same order of magnitude in each bone. The percentage increase in calcium was very small, much less than that of the controls of the same sex and of the males of the same group; but was an increase, while in the thyroparathyroidectomized females a decrease obtained.

The percentage increase in magnesium was almost the same as that of the controls, less than that of the males of the same group, much greater than that of the thyroparathyroidectomized females, and much greater than that of calcium and phosphorus.

The percentage increase in phosphorus was not large, much less than that of the controls of the same sex and the males of the same group, greater than that of calcium, but an increase, while in the thyroparathyroidectomized females there was a decrease.

The inhibitory effects of parathyroid deficiency were much less intense than were those of thyroid (thyroparathyroid) deficiency, but the same general type of interconstituent differentiation was present in both as was present in the males. The same holds true for the intersexual differences.

It is thus evident that the lack of parathyroid function has initiated a change in the nature of the ash of both bones of the females. The result of this disturbance as seen from the data in Table II was the production of an ash having a percentage of calcium not only lower than that of the controls of the same age, but also lower than that of the thyroparathyroidectomized females, and with a higher percentage of magnesium and phosphorus. Hence the course of the differentiation was the same in the parathyroidectomized and in the thyroparathyroidectomized rats, but quantitatively greater.

The course of differentiation in magnesium and phosphorus was the same as in the male parathyroidectomized rats and was similarly much greater than that obtaining in the thyroparathyroidectomized rats. Hence, the interpretation of the drift in the latter as a parathyroid deficiency effect is strengthened.

The perfectly definite decrease in the percentage of calcium of the ash in both bones of the female thyroparathyroidectomized and parathyroidectomized rats, differing as it does from the stability shown in the males is in general and in part explainable on the basis of the inherent difference in sensitivity of the gonads of the two sexes to the loss of the thyroid (thyroparathyroid) or parathyroid functions.

It was found and is reported in another place (19) that the growth of the testes of the male thyroparathyroidectomized rats, the analyses of the ash of the bones of which are the subject of this paper, was 70.0 per cent of the normal, while the body weight growth was 31.0 per cent; and the growth of the testes of the parathyroidectomized rats was 109.2 per cent, and the body weight 57.1 per cent of the normal. In the female thyroparathyroidectomized rats, on the other hand, the ovaries lost 236.3 per cent of what they should have gained, while the body weight decrease was only 28.2 per cent; and in the parathyroidectomized females the ovary loss was 90.3 per cent, while the body weight gain was 20.7 per cent of the normal.

These values show that the loss of the thyroid apparatus or the parathyroids did not cut down the growth of the testes as compared with the growth of the body as a whole, while it not only stopped the growth of the ovaries but induced marked retrogression and loss of substance.

It is well known that a disturbed ovarian function frequently accompanies osteomalacia, a disease characterized by a diminished calcium and an increased magnesium content of the bones (20). It is the opinion of many that the disease is due to ovarian dysfunction (21). Freund and Lockwood (22) found that ovariectomy in osteomalacia was followed by an increase in the negative calcium balance, and experiments have shown that "transplantation" of the ovaries produces a low calcium content of the bones, while "castration" is followed by no distinct change (23). In addition the results reported in this and the previous paper (1) show that the humerus and femur of the normal female rat contain a higher percentage of ash and calcium in the ash than do these bones in the males.

Knowing as I do that the lack of the thyroid (thyroparathyroid) or parathyroid functions reverberates throughout the organism and causes severe and differential inhibitions of growth (24, 25), and in view of the lack of systematic data on the effects of gonadectomy on the composition of bone ash, I would hesitate to say that the decalcification of the bones in the thyroparathyroidectomized and parathyroidectomized females was a direct consequence of the ovarian degeneration.

On the other hand, I do believe that the specific sex difference in response of the gonads to thyroid (thyroparathyroid) and parathyroid lack, the normally higher percentage of calcium in the ash of the female bones, and the observations on osteomalacia point unmistakably to the retrogression of the ovaries as a factor, probably the determining factor, in the decalcification reported here.

That it is a combination of ovarian dysfunction and parathyroid deficiency is suggested by the fact that it is in the parathyroidectomized rats that the effect is most marked. Since the ovarian retrogression is much less in the parathyroidectomized rats than in the thyroparathyroidectomized rats it is possible that the parathyroid deficiency is primary, the ovarian dystrophy

being the secondary but determining factor in the combination for decalcification. Such being the case it is probable that the decalcification of the thyroparathyroid bones is primarily a parathyroid effect, as was concluded for the increased percentage of magnesium and phosphorus from other premises.

The findings of McCrudden (20) that the bones in osteomalacia contain a higher percentage of magnesium than the normal, when correlated with my findings in parathyroidless rats and the above are suggestive, but the data here or elsewhere reported do not justify extension of the analysis.

SUMMARY AND CONCLUSIONS.

A study is made of the changes in the chemical composition of the ash of the humerus and femur of male and female albino rats during the growth period from 100 to 150 days of age and the effect of thyroparathyroidectomy and parathyroidectomy thereon.

There is no definitely detectable differentiation in ash composition occurring in the humerus and femur of normal rats during the 50 day period of observation.

The two bones exhibit a uniformity of percentage increase in the three ash constituents determined (calcium, magnesium, and phosphorus) and a uniformity of percentage composition of the ash. Since this holds for every group it indicates that the chemical processes concerned with ossification (the deposition of the calcium, magnesium, and phosphorus compounds characteristic of osseous tissue) are identical for serially homologous bones, notwithstanding sex differences in intensity, or qualitative alterations of activity due to thyroid (thyroparathyroid) or parathyroid deficiency.

When the thyroid apparatus is removed the ossification ability of the bones is markedly reduced so that there is produced a bone of both absolutely and relatively less ash content. This is considered as largely due to the loss of the stimulus to general growth provided by the thyroid. In both bones of both sexes there is a slight shift in the nature of the ash deposited. The direction of this shift is towards a higher percentage of magnesium and phosphorus than in the normal. This change in ash composition is believed to be an effect of the parathyroid deficiency

since it is shown to a much more marked degree in the bones of the rats which had only their parathyroid glands removed.

No change in the percentage of calcium of the ash was caused by thyroparathyroidectomy in the male rats.

When the parathyroid glands alone were removed the shift in magnesium and phosphorus percentage of the ash was as described above, and in the males no change in percentage of calcium was obtained.

It is therefore clear that although the calcium-retaining ability of the organism is reduced by the lack of function of both the thyroid and the parathyroid glands as shown by the greater excretion of this element, its lower concentration in the blood and the decreased ossification of the bones, the process of calcification of the ash, is undisturbed. This is evidence that the formation of a bone ash of normal calcium content is not directly related to thyroid or parathyroid function.

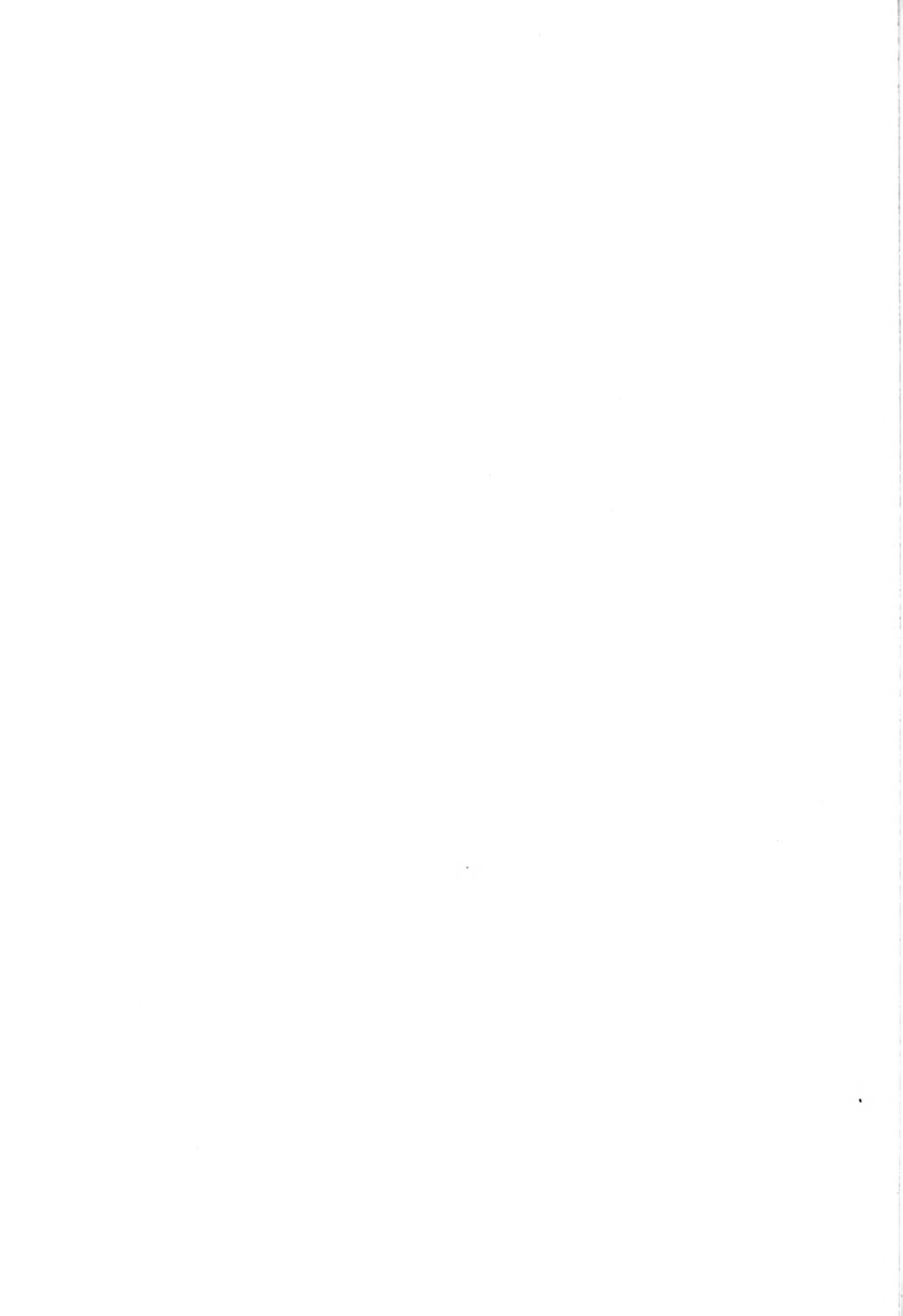
A differentiation between these two phases of calcium utilization by the organism and their relation to thyroid and parathyroid function is of importance.

The ash of both bones of the parathyroidectomized female had a lower percentage of calcium than the normal. The same was true but to a lesser degree of the ash of the thyroparathyroidectomized females. It is therefore probable that the lower percentage of calcium in the ash of thyroparathyroidectomized rats is to be associated with parathyroid rather than thyroid deficiency. Data are presented which justify the belief that the decrease in calcium percentage of the ash of the bones of the female thyroparathyroidectomized and parathyroidectomized rats is due to a combination of parathyroid deficiency and ovarian dystrophy, the latter probably being the determining factor acting upon a substratum already weakened as to its calcium-retaining ability by the toxemia produced by the lack of the function of the parathyroids.

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EXPERIMENTS ON THE UTILIZATION OF THE CALCIUM OF ALMONDS BY MAN.

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That there is little difference in the efficiency with which the adult human subject utilizes the calcium of carrots compared with that of milk has been shown by one of the authors (1) in a previous experiment. Since then Sherman and Hawley have found that in the case of young children the calcium of vegetables is not as readily stored as the calcium of milk (2), but Blatherwick and Long (3) found in two experiments with adult women on a diet in which about half the calcium came from an assortment of vegetables that there was considerable storage with an intake of from 0.5 to 0.75 of a gm. of calcium per day.

In the present study the almond was used as the main source of calcium, the general plan of the experiment being similar to that on the utilization of calcium in the carrot. Three new studies on the comparative utilization of calcium in milk were also made. Twelve healthy young women served as subjects. The diet taken by each was uniform throughout the 15 days of the experiment. Urine and feces were analyzed in 3 day periods. Calcium was determined by McCrudden's method. The almonds were blanched, air-dried, finely ground, and made into wafers with white of egg. These when baked were crisp and easily eaten. One attempt was made to use almonds from which a considerable part of the oil had been expressed, hoping to facilitate the digestion of the diet by decreasing the percentage of fat. This, however, proved impractical.

In these, as in the earlier experiments, the diet was planned to yield calcium equivalent to the estimated minimal requirement of each subject (0.0065 gm. per kilo of body weight). We

found, however, in our first and third series (Experiments 1, 2, 3, 8, and 9 in Table V) that our actual intake was lower than the original estimate, the calcium content of the almonds being only a little more than half the amount expected (0.132 per cent instead of 0.239 per cent). In the second series (Experiments 4 to 7) we anticipated a similar composition of our almonds,

TABLE I.
Daily Food Intake in Grams per Capita.

Food material.	Experiment No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Almonds, blanched	104	136	136	222	202	202	213	—	—	—	—	—
Apples, edible portion	119	159	159	95†	636	636	736	318	318	260	250	250
Beef, lean round	74	98	98	64	128	64	64	128	128	125	125	100
Butter	29	39	39	—	—	13	13	26*	26*	75	50	67
Crackers	73	96	96	24	60	48	48	18	48	—	—	—
Egg white	37	49	49	100	150	150	200	50	50	—	—	—
Sugar	75	100	100	—	50	—	50	75†	75†	—	50	—
Coffee	—	—	—	1.3	—	1.3	—	—	—	—	—	—
Ca(H ₂ PO ₄) ₂ ·H ₂ O	—	—	—	0.66	—	—	—	—	—	—	—	—
Almond press-cake	—	—	—	—	—	—	—	175‡	175‡	—	—	—
Bread, white	—	—	—	—	—	—	—	—	—	110	175	150
Milk, whole	—	—	—	—	—	—	—	—	—	193	258	183
Rice	—	—	—	—	—	—	—	—	—	80	90	75
Tomato juice	—	—	—	—	—	—	—	—	—	100	125	100
G. Washington coffee	—	—	—	—	—	—	—	—	—	—	25	—

* Periods I to II. Reduced to 13 gm., Periods III to V.

† Periods I to II. Reduced to 50 gm., Periods III to V.

‡ Periods I to II. Changed to 180 gm. of almonds, Periods III to V.

although they were a different lot, but found the calcium yield almost as high as our original expectation (0.223 per cent). In the experiments in which these almonds were used, the total intake was consequently higher than we had intended. However, intake of calcium is in all cases within the range of normal human requirement as reported by Sherman (4).

The total diet in the almond experiment consisted of almonds, apples, lean beef, butter, crackers, egg white, sugar, and in two cases, coffee. The proportion of the total calcium derived from

TABLE II.
Calcium Content of Foods Used in Diets.

Food material.	Experiment No.	Period.	Calcium.
			<i>per cent</i>
Almonds, blanched	1-3	I-V	0.132
	4-7	I	0.221
		II-III	0.223
		IV-V	0.212
		III-V	0.138
Almond press-cake	8, 9	I-II	0.141
Apples	1-7, 11-12	I-V	0.005
	10	I-V	0.006
	8, 9	I-V	0.004
Beef, lean round	1-3	I	0.013
		II	0.039
		III	0.034
		IV	0.013
		V	0.021
	4-7, 11-12	I	0.015
		II	0.008
		III-IV	0.011
		V	0.007
	8-9	I-II	0.005
		III-V	0.004
	10	I-II	0.018
		III-V	0.014
Bread, white	10	I-V	0.035
	11-12	I-V	0.028
Butter	1-3	I-V	0.019
	6, 7, 11, 12	I-V	0.016
		I-V	0.014
		I-V	0.020
Calcium phosphate ($\text{Ca}(\text{HPO}_4)_2 \cdot \text{H}_2\text{O}$)	4	I-V	16.0
Coffee	4, 6	I-V	0.298

TABLE II—*Concluded.*

Food material.	Experiment No.	Period.	Calcium. <i>per cent</i>
Crackers.			
Unedas.	1-3	I-V	0.021
Saltines.	4-7	I-V	0.023
Unedas.	8-9	I-V	0.025
Egg white.	1-3	I-V	0.010
	4-7	I-V	0.012
	8-9	I-V	0.015
Milk.	10	I-V	0.118
	11-12	I-V	0.115
Rice.	10	I-V	0.008
	11-12	I-V	0.006
Tomato juice.	10	I-V	0.003
	11-12	I-V	0.007

almonds ranged from 73 to 86 per cent, and is stated for each subject in Table V. In the milk experiments, white bread, whole milk, rice, tomato juice, and in one case G. Washington coffee were used (the same diet as in the milk experiments previously reported (1)). Milk furnished from 70 to 74 per cent of the total calcium. Distilled water only was used throughout these experiments. The daily food intake and calcium content of the foods used are shown in Tables I and II.

Digestibility of Almond Diet.

The digestibility of the almond diet was investigated separately, there being some doubt as to how efficiently a diet so high in fat would be absorbed. Five young women served as subjects, two of whom were also subjects in the calcium balance experiment (H. G., Experiment 6 and J. R., Experiment 7). The diet in this experiment was as shown in Table III.

Qualitative tests for carbohydrates in the feces indicated that the amount was small if any. Our experiences with rolled oats led us to believe that more was to be learned about the fate of

TABLE III.
Almond Diet Used in Digestion Experiment.

Food.	Weight of food.	Amounts per capita per day.			
		Nitrogen.	Protein (N \times 6.25).	Fat.	Total calories.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Almonds, blanched	180	6.98	43.63	105.55	1,165
Apples, edible portion	318	0.20	1.25	1.59	200
Beef, lean round	128	4.30	26.88	15.64	261
Sugar	50				200
Butter	13	0.02	0.13	11.05	100
Crackers (saltines)	48	0.74	4.63	6.10	207
Egg white	50	0.94	5.88	0.10	25
Total		13.18	82.40	140.03	2,158

TABLE IV.
Average Daily Output of Protein and Fat in Feces in Digestion Experiment with Almond Diet.

Subject.	Nitrogen.	Protein (N \times 6.25).	Fat.	Coefficients of digestibility.	
				Protein.	Fat.
	<i>gm.</i>		<i>gm.</i>		
H. G.	0.97	6.04	11.65	92.6	91.6
M. L.	1.18	7.38	13.55	91.1	90.3
C. M.	1.77	11.04	12.83	86.6	90.8
F. O.	2.47	15.43	12.70	81.2	90.9
J. R.	1.34	8.38	11.50	89.8	91.8
Average	1.55	9.65	12.45	88.3	91.1

TABLE V.
Average Daily Intake and Output of Calcium on Almond Diet.

Experiment No.	Subject.	Body weight.	Ca from almonds.	Per kilo of body weight.		
				Intake.	Output.	Balance.
		<i>kg.</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	B.H.	45	74	4.1	5.1	-1.0
2	M.F.	60	73	4.1	5.6	-1.5
3	M.K.	60	73	4.1	4.9	-0.8
4	D.H.	75.8	73	8.8	7.7	+1.1
5	E.B.	55	85	9.4	9.4	0.0
6	H.G.	55	86	9.3	11.6	-2.3
7	J.R.	58	85	9.4	11.1	-1.7
8	B.B.	52.7	86	5.5	10.1	-4.6
9	H.C.	50.5	86	5.7	7.1	-1.4

the almonds (5) from a study of protein and fat. The utilization of these foodstuffs was almost as high as for oatmeal mush in a simple mixed diet.

Coefficients of Digestibility of Almond and Rolled Oats (Oatmeal Mush) Diets.

Food.	Protein.	Fat.
	<i>per cent</i>	<i>per cent</i>
Almond.....	88.3	91.1
Oatmeal.....	89.9	91.8

The diet was taken for 3 days, the feces being marked off by carmine; collected feces were dried daily on a water bath after being rubbed to a paste with alcohol slightly acidified with hydrochloric acid, combined, and ground for analysis. The results are shown in Table IV. There was no evidence, therefore, of serious loss

TABLE VI.
Average Daily Intake and Output of Calcium on Milk Diet.

Experiment No.	Subject.	Body weight.	Ca from milk.	Per kilo of body weight.		
				Intake.	Output.	Balance.
		<i>kg.</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
10	B.B.	54	70	6.0	6.4	-0.4
11	G.C.	62.5	74	6.4	6.3	+0.1
12	C.M.	45	71	6.6	7.4	-0.8
13*	E.B.	54	70	7.1	6.0	+1.1
14*	R.E.	56	70	6.8	5.3	+1.5

* Previously reported, *cf.* Rose (1).

of calcium in feces through failure of digestion. In one case (B. B., Experiment 8) we felt that there was some likelihood of poor absorption, her output of calcium being extremely high. That her low intake was in part the cause of her high negative balance was shown by her output on the milk diet (Experiment 10), in which a higher intake practically resulted in equilibrium.

The average daily balance of calcium in nine experiments on the almond diet is shown in Table V.

To allow ample time for adjustment to the experimental diet, the figures for the first period have in each case been excluded from the averages. With two exceptions (H. G., Experiment

6 and B. B., Experiment 8) the subjects were practically in calcium equilibrium. One of these (B. B.) has already been referred to. In the other case the digestion experiment (see Table IV) showed even better utilization than the average of the group, so that this is clearly a case of inadequate supply.

TABLE VII.

*Summary of Calcium Balance Experiments.
Average Daily Intake, Output, and Balance per Kilo of Body Weight.*

Experiment No.	In- take.	Out- put.	Bal- ance.	Ratio of urinary to fecal calcium.
Period I. Almond diet.				
1, 2, 3	4.1	5.2	-1.1	1:8.8
4, 5, 6, 7	9.2	9.9	-0.7	1:34.6; 1:6.9; 1:35.5; 1:22.4
8, 9	5.6	8.6	-3.0	1:8.9
Period II. Milk diet.				
10, 11, 12, 13, 14	6.6	6.3	+0.3	1:3.4
Period III. Carrot diet.				
15, 16, 17	5.7	5.4	+0.3	1:3.4
18	6.3	4.3	+2.0	1:2.3
Period IV. Vegetable diet (Blatherwick and Long).				
19	13.1	9.5	+3.6	1:3.4
20	8.7	7.8	+0.9	1:3.8

The utilization of calcium on all the milk experiments which we have conducted (three new ones and two formerly reported) are shown in Table VI. In these, equilibrium was practically established on 6 to 7 mg. per kilo of body weight.

We are now able to compare data on three types of diet of our own (milk, almond, and carrot) and the two experiments reported by Blatherwick and Long on a mixed vegetable diet. These are briefly summarized in Table VII. The protocols of the present experiments are given in full in Table VIII.

TABLE VIII.
Intake and Output of Calcium in 3 Day Periods.

Experiment No.	Subject.	Period.	Average daily intake.	Average daily output.			Balance.
				Urine.	Feces.	Total.	
Almond diet.							
1	B.H.		gm.	gm.	gm.	gm.	gm.
		II*	0.197	0.037	0.157	0.194	+0.003
		III	0.195	0.018	0.143	0.161	+0.034
		IV	0.177	0.044	0.286	0.330	-0.153
		V	0.183	0.010	0.182	0.192	-0.009
Average		II-V	0.188	0.027	0.192	0.219	-0.031
2	M.F.	I	0.233	0.021	0.144	0.165	+0.068
		II	0.255	0.028	0.311	0.339	-0.084
		III	0.253	0.039	0.275	0.314	-0.061
		IV	0.233	0.015	0.246	0.261	-0.028
		V	0.241	0.063	0.374	0.437	-0.196
Average		II-V	0.246	0.036	0.302	0.338	-0.092
3	M.K.	I	0.233	0.019	0.406	0.425	-0.192
		II	0.255	0.036	0.263	0.299	-0.044
		III	0.253	0.030	0.268	0.298	-0.045
		IV	0.233	0.032	0.311	0.343	-0.110
		V	0.241	0.019	0.208	0.227	+0.014
Average		II-V	0.246	0.029	0.263	0.292	-0.046
4	D.H.	I	0.676	0.020	0.417	0.437	+0.239
		II	0.676	0.018	0.482	0.500	+0.176
		III	0.677	0.017	0.460	0.477	+0.200
		IV	0.653	0.017	0.578	0.595	+0.058
		V	0.650	0.013	0.739	0.752	-0.102
Average		II-V	0.664	0.016	0.565	0.581	+0.083
5	E.B.	I	0.529	0.042	0.570	0.612	-0.083
		II	0.524	0.061	0.401	0.462	+0.062
		III	0.527	0.057	0.541	0.598	-0.071
		IV	0.505	0.060	0.480	0.540	-0.035
		V	0.500	0.084	0.381	0.465	+0.035
Average		II-V	0.514	0.066	0.451	0.516	-0.002

* No analysis made for Period I.

TABLE VIII—*Continued.*

Experiment No.	Subject.	Period.	Average daily intake.	Average daily output.			Balance.
				Urine.	Feces.	Total.	
Almond diet—Continued.							
6	H.G.	I	gm. 0.523	gm. 0.023	gm. 0.451	gm. 0.474	+0.049
		II	0.522	0.017	0.646	0.663	—0.14
		III	0.524	0.011	0.518	0.529	—0.005
		IV	0.502	0.013	0.453	0.466	+0.036
		V	0.499	0.029	0.866	0.895	—0.396
Average		II-V	0.512	0.018	0.621	0.638	—0.127
7	J.R.	I	0.554	0.005	0.581	0.585	—0.031
		II	0.554	0.034	0.756	0.791	—0.237
		III	0.555	0.029	0.571	0.600	—0.045
		IV	0.532	0.024	0.655	0.679	—0.147
		V	0.530	0.022	0.478	0.500	+0.029
Average		II-V	0.543	0.027	0.615	0.643	—0.100
8	B.B.	II*	0.289	0.037	0.535	0.572	—0.283
		III	0.288	0.044	0.447	0.491	—0.203
		IV	0.288	0.056	0.463	0.519	—0.231
		V	0.288	0.043	0.499	0.542	—0.254
Average		II-V	0.288	0.045	0.486	0.531	—0.243
9	H.C.	II*	0.289	0.063	0.305	0.368	—0.079
		III	0.288	0.053	0.222	0.275	+0.013
		IV	0.288	0.014	0.440	0.454	—0.166
		V	0.288	0.046	0.284	0.330	—0.042
Average		II-V	0.288	0.044	0.313	0.357	—0.069
Milk diet.							
10	B.B.	I	0.328	0.045	0.356	0.401	—0.072
		II	0.328	0.056	0.303	0.359	—0.031
		III	0.323	0.068	0.259	0.327	—0.004
		IV	0.323	0.058	0.297	0.355	—0.032
		V	0.323	0.070	0.280	0.350	—0.027
Average		II-V	0.324	0.063	0.285	0.348	—0.024

TABLE VIII—*Concluded.*

Experiment No.	Subject.	Period.	Average daily intake.	Average daily output.			Balance.
				Urine.	Feces.	Total.	
Milk diet—Continued.							
11	G.C.	I	gm. 0.407	gm. 0.039	gm. 0.362	gm. 0.401	+0.006
		II	0.398	0.041	0.221	0.263	+0.135
		III	0.401	0.034	0.314	0.348	+0.053
		IV	0.401	0.134	0.302	0.436	−0.035
		V	0.396	0.101	0.415	0.516	−0.120
		Average	II-V	0.399	0.078	0.313	0.391
12	C.M.	I	0.302	0.078	0.553	0.631	−0.329
		II	0.295	0.071	0.247	0.318	−0.023
		III	0.298	0.103	0.235	0.338	−0.040
		IV	0.298	0.072	0.280	0.352	−0.054
		V	0.294	0.090	0.240	0.330	−0.036
		Average	II-V	0.296	0.084	0.251	0.335

CONCLUSIONS.

In experiments on adult women in which about 73 per cent of the calcium of the diet was derived from almonds, there was a daily output of from 5 to 6 mg. of calcium per kilo of body weight on an intake of 4.1 mg. per kilo, indicating that calcium equilibrium could be secured about as efficiently as when milk furnished approximately the same proportion of the calcium (70 per cent) or when carrots furnished either 55 or 85 per cent of the total; namely 5 to 6 mg. per kilo.

When the almonds contributed 85 to 86 per cent of the calcium, the amount necessary for equilibrium was increased. One subject was in equilibrium on 9.4 mg. per kilo, and another had a shortage of 1.4 mg. on an intake of 7.1 mg. per kilo, but the output of three other cases indicated the need of from 10 to 12 mg. Hence it would seem that from 8 to 12 mg. per kilo are required when almonds furnish all but 14 to 15 per cent of the total calcium.

These returns are similar to those of Blatherwick and Long on a mixed vegetable diet in which about 50 per cent of the calcium was derived from vegetables with an indication of equilibrium on

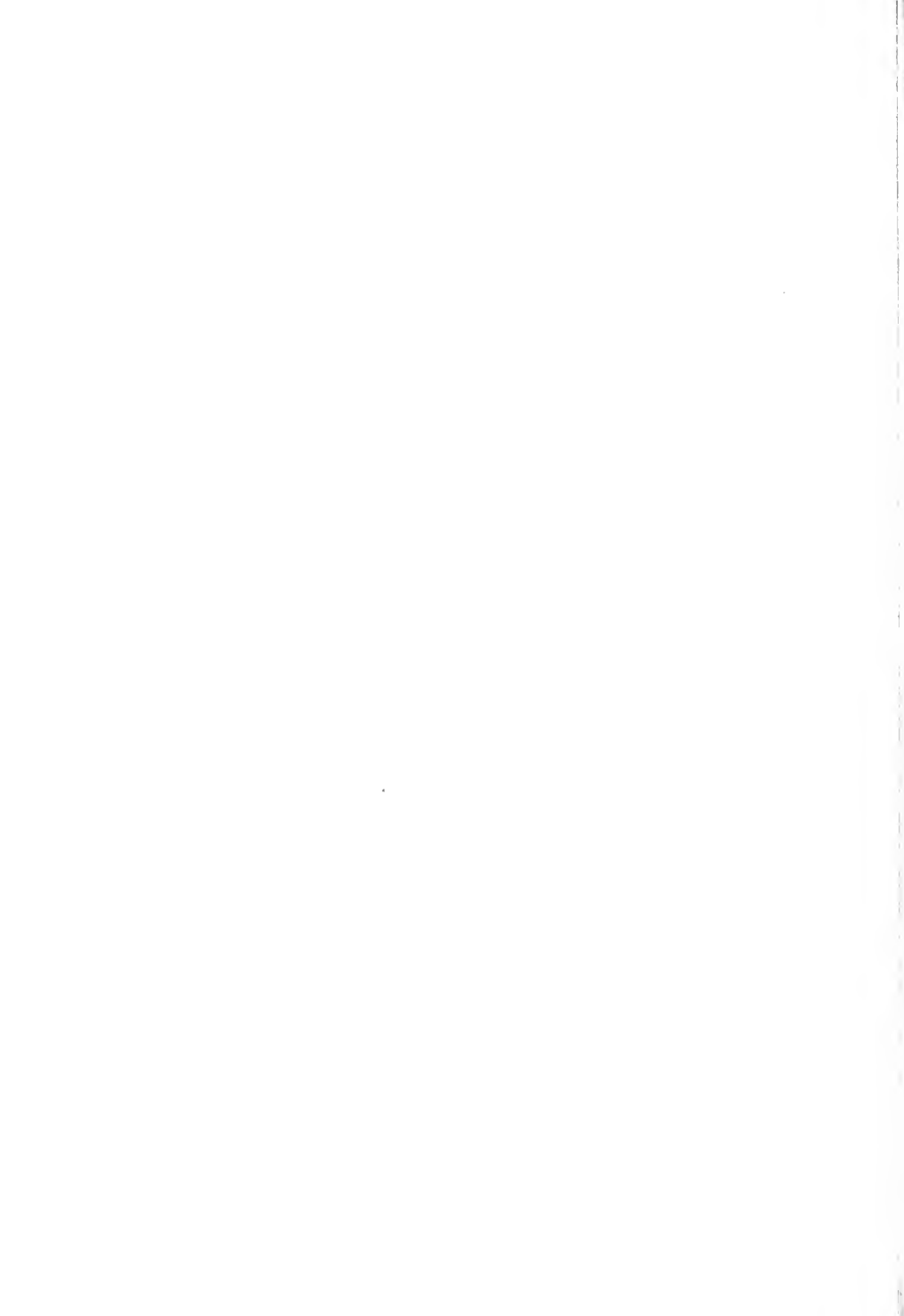
8 to 10 mg. per kilo. It seems quite likely that the very high fat content of the "86 per cent" almond diet (140 gm. per capita) increased the loss of calcium, though a digestion experiment with five subjects showed that the coefficients of digestibility for protein and fat were only 4 and 3 per cent less, respectively, than the average allowances for loss on an ordinary mixed diet.

No light seems to be thrown on the situation by examination of the ratio between urinary and fecal calcium. On the milk and carrot diets they are narrow, approximately 1 (urinary) to 3.4 (fecal). On Blatherwick and Long's mixed vegetable diet they were 1:3.4 and 1:3.8. On the almond they ranged from 1:6.9 to 1:34.6, the widest ratio occurring in the case where there was the greatest storage of calcium.

On the whole it would appear that almonds contribute efficiently to the calcium of the diet, but when a very high proportion of the calcium comes from almonds the conditions in the digestive tract may not be quite so favorable for economical utilization as when carrots or milk form the main source of the calcium.

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ON THE PREPARATION OF DIACETONE GLUCOSE.

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Diacetone glucose serves as starting material for the preparation of many partially substituted sugars. The details of its preparation have been repeatedly undergoing modification. Originally, ordinary α -glucose served for its preparation. The process, however, was very laborious and the yield was rather small for the reason that prior to condensing with acetone, the sugar was converted into its methylacetal, as the α -glucose itself was supposedly too insoluble to react with acetone. Fischer therefore suggested the use of β -glucose, the more soluble form of this sugar for condensation with acetone. The use of absolutely dry and alcohol-free acetone was also urged for the preparation of diacetone glucose.

Since we were in need of comparatively large quantities of the condensation product, we resolved to make a new attempt to prepare diacetone glucose from the α isomer. As will be seen from Table I diacetone glucose can be advantageously prepared from this isomer. It is true that the yield of diacetone glucose per unit weight of the employed sugar is at a maximum when the conditions of Fischer are observed; namely, when β -glucose and pure dry acetone are employed for the reaction. If, however, economy of time is considered, then per unit time, more product is obtained when diacetone is made from the common form of glucose even with ordinary commercial acetone, and surely when pure and dry acetone is employed. From the view of the cost of materials per unit weight of the diacetone glucose obtained and from the view-point of purity of the resulting material, no one method offers any advantage over the other.

In the course of preparation of diacetone glucose, there always remains a residue. Fischer and Rund¹ mentioned this fact in describing the method of preparation from β -glucose. They stated that the residue consisted of the α isomer. In our experience, however, the residue consisted of the original form used for condensation. This is important to bear in mind when one is inclined

TABLE I.

Sugar in 10.0 gm. lots.	Acetone.	Residue.	Diacetone yield.	Average yield.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
β -glucose.	Anhydrous.	42	56	55
		42	55	
α -glucose.	“	60	39	36
		62	34	
β -glucose.	Ordinary.	49	45	42
		56	39	
α -glucose.	“	73	26	25
		68	24	

On one recrystallization from petroleum ether the substances by every process melted at 110°C.

to use the β form for the preparation of diacetone glucose, for the reason that the residue can be reemployed.

¹ Fischer, E., and Rund, C., *Ber. chem. Ges.*, 1916, xlix, 93.

ON MONOACETONE BENZYLIDENE GLUCOSE.

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In a previous publication,¹ it was reported that two different phosphoric esters of glucose were obtained depending upon the sugar derivative used for condensation with phosphorus oxychloride. In the substance obtained from diacetone glucose, the phosphoric acid radicle seemed to be attached to one of the carbon atoms other than 1 and 6. In the product obtained from monoacetone monobenzylidene glucose, the phosphoric acid radicle was more firmly attached to the sugar molecule and from this it could be concluded that it was linked to carbon atom 6. Two alternative explanations could be given to this observation. Either diacetone glucose and monoacetone benzylidene glucose have their free hydroxyl in a different position, or in monoacetone benzylidene glucose, the hydroxyl in position 6 is set free before the benzaldehyde radicle is completely liberated (possibly the benzaldehyde radicle migrates from position 5 and 6 to others). The structure of diacetone glucose has been recently explained through the work of Karrer and Hurwitz,² Levene and Meyer,³ and Freudenberg and Brauns.⁴ It was then necessary to correlate the structure of monoacetone benzylidene glucose with that of diacetone glucose. This was accomplished. It was found that the two derivatives gave the identical monoacetone benzoyl glucose. Also, the same monomethyl glucose was obtained from diacetone glucose and from monoacetone benzylidene glucose. The two samples of monomethyl glucose had the same optical rotations and the same melting points. On oxidation, the monomethyl

¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

² Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

³ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liv, 805.

⁴ Freudenberg, K., and Brauns, F., *Ber. chem. Ges.*, 1922, lv, 3233.

glucose from monoacetone benzylidene glucose gave a crystalline product as did the monomethyl glucose prepared from diacetone glucose. Unfortunately, the supply of monoacetone benzylidene glucose was very small. The substance is much more difficult to prepare than diacetone glucose and the yield of methylsaccharicanhydride was too small for analysis. However, the melting point was at $207^{\circ}\text{C}.$, which is identical with that of 1,4-anhydro-3-methylsaccharic acid. Thus, the evidence adduced is sufficient to assume for monoacetone benzylidene glucose a structure analogous to that of diacetone glucose, and is to be regarded as 1,2-acetone-5,6-benzylidene glucose.

EXPERIMENTAL.

Monoacetone Benzylidene Glucose.—The method for preparing this substance as previously described by us was slightly modified.

50 gm. of monoacetone glucose were heated with 300 cc. of freshly distilled benzaldehyde and 100 gm. of anhydrous sodium sulfate in an oil bath at 170° for 5 hours. The reaction product was filtered from the sodium sulfate while still hot and concentrated under diminished pressure at $130^{\circ}\text{C}.$ (oil bath temperature). The product was cooled and poured into 1,000 cc. of cold petroleum ether, $30\text{--}60^{\circ}$. A sticky mass which solidified on stirring, settled out. This brownish mass was washed several times with cold petroleum ether and subsequently with a small quantity of ether, and then filtered. A practically colorless substance was obtained. It was recrystallized several times from 95 per cent alcohol and decolorized with norit. The petroleum ether solutions were concentrated and an additional yield of crude material was obtained in this manner. It was recrystallized from 95 per cent alcohol. The pure substance melted at 144° and analyzed as follows:

0.1052 gm. substance: 0.2408 gm. CO_2 and 0.0618 gm. H_2O .

$\text{C}_{16}\text{H}_{26}\text{O}_6$. Calculated. C 62.30, H 6.54.

Found. " 62.42, " 6.58.

Methyl Benzylidene Glucose and Its Hydrolysis to Methyl Glucose.—15 gm. of monoacetone benzylidene glucose were methylated with an excess of dimethyl sulfate and 30 per cent sodium hydroxide at 70° (water bath temperature). The reaction

product was extracted with chloroform and dried with anhydrous sodium sulfate. The chloroform was removed under diminished pressure. The syrup which remained was hydrolyzed.

For this purpose, it was dissolved in 75 cc. of 50 per cent alcohol, containing 0.4 per cent of hydrochloric acid, and heated in boiling water with reflux for 70 minutes. The solution was cooled and the hydrochloric acid removed with silver carbonate. The solution was then treated with hydrogen sulfide, filtered, decolorized with norit, and concentrated to a syrup under diminished pressure. The residue was taken up in absolute alcohol and again evaporated to dryness in order to remove traces of water. This operation was repeated several times. The syrup was then taken up in warm methyl alcohol and allowed to stand in a desiccator over sulfuric acid. On standing, crystals of methyl glucose separated. These had the following optical rotations in water.

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 1} = \frac{+1.03^\circ \times 100}{1 \times 1} = +103^\circ \quad [\alpha]_D^{20} = \frac{\text{Final.}}{1 \times 1} = \frac{+0.57^\circ \times 100}{1 \times 1} = 57^\circ$$

The substance melted at 156-157°C.

Oxidation of Methyl Glucose.—0.450 gm. of the sugar was dissolved in 25 cc. of 50 per cent nitric acid, allowed to stand at room temperature for 42 hours, and then evaporated in a clock glass on a boiling water bath, as described in a previous publication.³ The residue was taken up in a mixture of ether and acetone. The solution was filtered and allowed to evaporate spontaneously. A small crystalline deposit formed. It was filtered and washed with a mixture of acetone and ether. The crystals darkened at 190° and melted at 207°C.

Monoacetone Benzoyl Glucose.—15 gm. of monoacetone benzyldene glucose were added to a solution of 15 cc. of pyridine and 7.5 of benzoyl chloride. The temperature rose slightly and all the sugar dissolved. No pyridine hydrochloride separated and the solution was kept at 45° for 24 hours. The product was then dissolved in chloroform and washed with very dilute sulfuric acid, dilute sodium bicarbonate, and water. The chloroform solution was dried with anhydrous sodium sulfate and concentrated under diminished pressure to a very small volume and poured into an excess of petroleum ether. A crystalline product was obtained

which was recrystallized from methyl alcohol. This melted at 198° . Mixed with monoacetone benzoyl glucose obtained from diacetone glucose, it melted at the same temperature. The substance analyzed as follows:

0.1038 gm. substance: 0.2262 gm. CO_2 and 9.060 gm. H_2O .
 $\text{C}_{16}\text{H}_{20}\text{O}_7$. Calculated. C 59.23, H 6.22.
 Found. " 59.42, " 6.55.

ON EPICHTOSAMINE PENTACETATE.

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The configuration of carbon atom 2 in nitrogenous sugars and their corresponding sugaracids remains a matter of speculation. In a previous publication¹ some analogies between chitosaminic and mannonic acids and between chondrosaminic and talonic acids were pointed out. Thus, on the basis of these analogies, one could surmise that chitosamine had the configuration of 2-amino-mannose and chondrosamine that of 2-aminotalose. Very recently however, Irvine and Earl² have pointed out that the difference between the molecular rotations of the α and β isomers of glucosamine was identical with the difference between the glucoses and therefore concluded that chitosamine had the configuration of glucosamine. It cannot be denied that the argument of Irvine and Earl may be correct. The possibility that chitosamine may have the configuration of 2-aminoglucose was never denied. The theory, however, is in need of further proof. The properties of glucose and of chitosamine which Irvine compares are those associated with the properties of carbon atom 1. In glucose and in chitosamine, carbon atom 1 functions regularly; in mannose, it functions irregularly. To this argument of Irvine and Earl may be added the fact that carbon atom 1 in epichitosamine hydrochloride, similarly to mannose, functions irregularly. Thus, on the basis of the conduct of carbon atom 1, one might be justified in grouping chitosamine with glucose and epichitosamine with mannose. However, it is not excluded that in the nitrogenous sugars carbon atom 1 functions differently than in ordinary sugars.

¹ Levene, P. A., Hexosamines, their derivatives, and mucins and mucoids, Monograph of The Rockefeller Institute for Medical Research, No. 18, New York, 1922.

² Irvine, J. C., and Earl, J. C., *J. Chem. Soc.*, 1922, cxxi, 2376.

Levene and Meyer³ have already described a peculiar behavior of this carbon atom in the epiglucosamine of Fischer, Bergmann, and Schotte. In the present paper, we wish to point out another unusual peculiarity. Both in a sugar and in a sugar acid, the direction of the rotation of carbon atom 2 may be determined either from the rotations of a pair of epimeric monocarboxylic sugar acids, or from the rotations of the α and β forms of the epimeric sugars. Taking glucose and mannose, it is possible to determine the rotation of carbon atom 2 from the rotations of the salts, amides, or phenylhydrazides of gluconic and mannonic acids. It is also possible to determine it from the values of the rotations of the α and β forms of glucose and mannose. Designating in each sugar the group of carbon atom 1 by A , that of carbon atom 2 by B and the remaining part of the molecule by C , one may represent the molecular rotation of glucose by the equation $A+B+C = m$ and that of mannose by $A_1-B+C = n$. When the molecular rotations of the sugars and of the carbon atoms 1 are known, the directions and the values of B can be readily determined from the equations, $(A+B+C)-(A_1-B+C) = m-n$ and $(A+B+C) + (A-B+C) = m+n$. In the system glucose and mannose either one of the two methods gives the direction to the right for carbon atom 2 of glucose and to the left for carbon atom 2 of mannose. The same result is obtained when instead of glucose and mannose, the pentacetates are used for calculation.

In the system chitosamine and epichitosamine one encounters a deviation from the rule. From the system chitosaminic and epichitosaminic acids carbon atom 2 is found to rotate to the left in chitosaminic and to the right in epichitosaminic acid. When the direction of the rotation of carbon atom 2 is determined from the values of the molecular rotations of chitosamine and epichitosamine, it is found that carbon atom 2 rotates to the left in epichitosamine and to the right in chitosamine. It is hardly probable that in the process of oxidation of a sugar to the corresponding monocarboxylic acid, the configuration of carbon atom 2 undergoes a change. Since a sugar and its monocarboxylic acid differ only by the character of carbon atom 1, it is more logical to assume that the differences in the direction of the rotations of the two

³ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1923, **lv**, 221.

carbon atoms 2 are brought about by differences in the structure of the carbon atoms 1. The condition is somewhat analogous to that of *l*-aspartic acid and its ester. The acid rotates to the right, whereas the ester rotates to the left. Thus, the task of settling the problem of allocation of the amino group in nitrogenous sugars is becoming rather more complicated than simplified. One is tempted to accept for chitosamine the configuration of 2-amino-glucose and for epichitosamine that of 2-aminomannose, inasmuch as the α and β isomers of chitosamine and glucose behave normally, and the corresponding forms of epichitosamine and mannose have an abnormal behavior. However, if this conclusion were accepted the case of epichitose would be puzzling. Epichitose, which has the structure of 2,5-anhydroglucose, resembles epichitosamine in regard to the function of its carbon atom 1. Both these sugars show no mutarotation and both possess levo-rotation. Thus, if one takes glucose for a standard of comparison, one may arrive with Irvine and Earl at the conclusion that chitosamine has the structure of 2-amino-glucose. On the other hand, taking 2,5-anhydroglucose as the standard, the conclusion may be reached that epichitosamine has the structure of 2-aminoglucose.

In the present communication, data are given for the determination of the direction of the rotation of carbon atom 2 in chitosamine and in epichitosamine. Chitosamine hydrochloride was fractionated by Irvine and Earl into the α and β isomers. Epichitosamine showed no mutarotation, and its specific rotation was very low. Because of these properties, it was uncertain whether epichitosamine had the glucosidic or the ordinary aldehydic structure. On the other hand, since the substance was levo-rotatory, it was thought that it might consist primarily of the β form, if it had the glucosidic structure. In view of the scarcity of the material, it was thought advantageous to prepare the pentacetate.

Regarding the pentacetates, experience has shown that in the *d* series, the β isomers are transformed into the α forms to the extent of not less than 90 per cent by heating in acetic anhydride, containing zinc chloride. In the *l* series, the reaction is reversed. β -Epichitosamine pentacetate was prepared and was found to have a molecular rotation of -3.890° in acetic anhydride. This form is to be regarded as the β isomer. The α form had a molecular

rotation of not less than $+19,450^\circ$. Hence, the difference of the rotations of the two isomers was not less than $+24,400^\circ$.

From the equation $[A + (\pm B) + C] - [A_1 + (\mp B) + C] = m - n$ (substituting for A its value $+17,965$, for A_1 the value $-12,643$, for m its value $36,400$, and for n the value $-3,890$) it follows that $B = \pm 4,841$; from the sum of factors of the same equation $C = +13,594$. Substituting these values, respectively, in equation $A + (\pm B) + C = 36,400$ and in equation $A_1 + (\mp B) + C = -3,890$, a positive value is obtained for B (carbon atom 2) of chitosamine and a negative value for epichitosamine.

EXPERIMENTAL PART.

Several lots of epichitosamine hydrochloride, each of 5.0 gm., were taken up in 27.0 cc. of acetic anhydride and 27.0 cc. of pyridine and allowed to stand 24 hours at 37°C . and 24 hours at room temperature. The reaction product was transferred into a mixture of ice and water and the pentacetate shaken out with chloroform. The chloroform solution was washed with water, dilute mineral acid, then with a cold solution of sodium bicarbonate acid, and finally with water. The chloroform solution was dried over anhydrous sodium sulfate and concentrated to dryness. The residue was dissolved in alcohol and evaporated to dryness. This operation was repeated several times. The final residue was crystallized from alcohol. For analysis, it was recrystallized from alcohol. The long white needles melted at $158\text{--}159^\circ\text{C}$. (corrected) and analyzed as follows:

0.1989 gm. substance: (Kjeldahl) 5.25 cc. 0.1 N acid.

0.1068 " " : 0.1934 gm. CO_2 and 0.0600 gm. H_2O .

$\text{C}_6\text{H}_5\text{NO}_5(\text{CH}_2\text{CO})_5$. Calculated. C 49.49, H 5.96, N 3.60.

Found. " 49.38, " 6.28, " 3.69.

The rotation of the substance in chloroform was:

$$[\alpha]_D^{20} = \frac{-0.54^\circ \times 100}{1 \times 3} = -18^\circ$$

Conversion of the β into the α Isomer.—A 3 per cent solution of the pentacetate in acetic anhydride, containing 1 per cent of zinc chloride, was kept in a thermostat at 40°C . and the rotation of the solution measured at intervals. The initial rotation was -0.30°

in a 100 mm. tube. The solution reached an equilibrium after 120 hours and then had a rotation of $+1.45^{\circ}$. In a second experiment, a 3 per cent solution of the substance was made in acetic anhydride, containing 3 per cent of zinc chloride, and the solution was refluxed on a boiling water bath. The initial rotation was $+0.30^{\circ}$ in a 100 mm. tube, and after 20 minutes the rotation was $+1.50^{\circ}$. It was not possible to make further readings as the solution darkened.

PREPARATION OF α -MANNOSE.

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Mannose has been prepared only in the form of its β isomer with a specific rotation of -17° . The specific rotation of the α form was obtained indirectly by Hudson and Yanovsky¹ and was found to be $+34$ or $+35^\circ$. The difference of the specific rotations was, therefore, $+52^\circ$, whereas in the case of the two glucoses, it is $+94^\circ$. The value for the two mannoses is exceptional. A value of the same magnitude was observed in the case of only two other sugars; namely, lyxose and rhamnose. Mannose occupies an exceptional position in other respects. The ratio of the initial and final solubilities of β -mannose in 80 per cent ethyl alcohol and in methyl alcohol is 1:5 and 1:5.6, respectively, and in that of the glucoses and of the galactoses, it is approximately 1:2. Also, it is peculiar that the difference of the specific rotations of two mannoses is equal to only 55 per cent of that of the two glucoses, whereas in the case of the pentacetates, the value of the difference is 80 per cent of that of the glucoses. In fact, the difference of the molecular rotations of the pentacetates of the mannoses (31,000) is very near to that of the galactoses (32,700). Because of the abnormal behavior of mannose and because this behavior has been made the basis of a theory of the structure of chitosamine,² it seemed desirable to prepare the unknown isomer of mannose. This has now been accomplished.

In the process of its preparation, another abnormality of mannose came to light. Under conditions when glucose and galactose appear in the β form, mannose crystallizes in the α form and *vice versa*. For glucose there exist two convenient methods for

¹ Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, 1917, xxxix, 1013.

² Irvine, J. C., and Earl, J. C., *J. Chem. Soc.*, 1922, cxxi, 2376.

the preparation of the β isomer. The one is that of Behrend,³ which consists in crystallizing glucose from a pyridine solution, the other is that of Hudson and Dale,⁴ and consists in crystallizing glucose from glacial acetic acid and alcohol at a low temperature (about 0°C.). A third much simpler and more economical method was discovered in the course of this work; namely, crystallization from aqueous ammonia and alcohol. Both glucose and galactose crystallize under this simple condition in the β form. Under all these three conditions, mannose crystallizes in the α form. This observation suggested the possibility that the configuration of the carbon atom 1 in β -glucose was the same as in α -mannose; namely, that the hydroxyl in both was to the right. Against such an assumption militates the behavior of their pentacetates. It is known that pentacetates of sugars may be transformed into the isomeric form by warming them in a solution of acetic anhydride containing zinc chloride. A scrutiny of all the known instances of transformation of pentacetates reveals the fact that in the *d* series, the β isomers are transformed into the α isomers, to the extent of over 90 per cent, whereas the transformation of the α form is minimal. In other words, in a solution of acetic anhydride and zinc chloride, the equilibrium mixture consists almost entirely of the α isomer. In the *l* series, the equilibrium is reversed. In this respect, the conduct of mannose conforms to all other known sugars. Hence, one may assume that the position of the hydroxyl on the carbon atom 1 in α - and β -mannose is the same as in the corresponding form of other simple sugars.

The specific rotation for α -mannose was found $+30^\circ$ in water and $+35^\circ$ in 80 per cent alcohol. This value is in complete agreement with the value calculated by Hudson and Yanovsky. Furthermore, the possession of the two forms permitted the establishment of the fact that the form with the specific rotation of -17° was free from the α form, and the mannose with a specific rotation $+35^\circ$ was free from the β form. This conclusion was reached on the comparison of the solubilities of the α and β isomers separately and of a mixture of the two isomers. The solubility of the mixture was equal to the sum of the solubilities of each of the isomers separately. Furthermore, from the initial and final

³ Behrend, R., *Ann. Chem.*, 1907, cccliii, 106.

⁴ Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1264.

solubilities of the α isomer, the specific rotation of the β form is calculated -16.5° . Hudson and Yanovsky find for the same form in 80 per cent alcohol -14.9° . The rotation of our pure α form was slightly higher. The differences are, however, within the limits of possible error. The evidence thus far adduced supports the view that the mannose with the specific rotation of $+35^\circ$ is the pure α isomer. However, mannose with the specific rotation of $+35^\circ$ may still contain a third isomer and yet on the basis of its initial and final solubility, it may give the correct value for the specific rotation of the β isomer and also give a combined solubility of the two forms as if they were pure α and β isomers.

A sufficient proof of the purity of each form should be based on the following considerations. It was shown independently by Hudson⁵ and by Lowry⁶ that mutarotation is a balanced reaction which follows the monomolecular form of the mass law and can be expressed by the equation

$$k_1 + k_2 = \log \frac{r_\infty - r_o}{r_\infty - r_t}$$

Furthermore, that the rate of transformation of each form into the other can be measured from the rate of their maximum solubilities by the equation k_1 (when the α form is considered) or k_2 (when the β form is under consideration) $= \log \frac{S_\infty - S_o}{S_\infty - S_t}$ (S stands for solubility). On the basis of these equations, it is possible, on the one hand, to determine the sum of $k_1 + k_2$ (from the mutarotation of either one of the two isomers) and on the other hand, independently k_1 and k_2 (from the rate of solubilities of each). Hence, the sum of k_1 and k_2 obtained independently should be equal to $k_1 + k_2$ obtained on the basis of mutarotation. It was intended to apply this test for the purpose of establishing the purity of the mannose with the specific rotation of $+35^\circ$ (in 80 per cent alcohol). It was surprising, however, to find that the value of $(k_1 + k_2)$ measured on the α form differed from that measured on the β form. The measurements were made in 80 per cent alcohol at several temperatures. In order to control the accuracy of our measurements, the muta-

⁵ Hudson, C. S., *J. Am. Chem. Soc.*, 1904, xxvi, 1065.

⁶ Lowry, T. M., *J. Chem. Soc.*, 1904, lxxxv, 1551.

rotations of α - and β -glucoses in solutions of 60 and 80 per cent ethyl alcohol were measured and found identical within the limits of error. Taking as a standard of comparison the coefficient at 12.5°C , it was of the order of magnitude of 0.00150 for the β form (recalculated for 20°C , it is 0.00333; this is in close agreement with the value of 0.00363 found by Hudson and Yanovsky), whereas the isomer gave the value for $k_1 + k_2 = 0.00240$ (recalculated for 20°C , $=0.00533$). One must add, however, that the entire range of change of the α form from the initial to final is rather small, so that only few measurements can be relied upon for calculating the velocity coefficient. However, since the difference between the two coefficients was constant, it may be regarded as real. It is important to compare their velocity coefficients in water. These, however, can be measured only at lower temperatures inasmuch as the α -mannose solution reaches equilibrium very rapidly. In view of the atmospheric humidity during the spring and summer months, this experiment has to be postponed until the winter. For the present, therefore, the possibility is not excluded that the mannose with a specific rotation of $+35^{\circ}$ (in a solution of 80 per cent alcohol) contains in addition to the α form still a third form.

This assumption may explain the fact that in the equilibrium mixture in 80 per cent alcohol and in methyl alcohol, the β form of mannose comprises only 18 per cent of the total, whereas in the cases of glucose and galactose, it comprises practically 50 per cent. It would also explain the fact that the value of the difference of the molecular rotations of the α - and β -mannoses is only 40 per cent of the normal value, whereas in the case of pentacetates, the value is 80 per cent of the normal.

SUMMARY.

1. A form of mannose was prepared which had a specific rotation of $+30^{\circ}$ in water and $+35^{\circ}$ in 80 per cent alcohol.
2. This form has the specific rotation calculated for α -mannose.
3. The conditions which lead to the formation of α -mannose are the same which lead to the formation of β -glucose and β -galactose.
4. The configuration of the carbon atom 1 in the α and β isomers, respectively, in glucose, galactose, and mannose is identical.

5. It is possible that the mannose with the specific rotation of $+35^\circ$ contains besides the α isomer still a third form.

6. A convenient and economical method is given for the preparation of β -glucose and of β -galactose.

EXPERIMENTAL PART.

Preparation of α -Mannose by the Pyridine Process.—25.0 gm. of α -mannose ($[\alpha]_D^{20} = -12^\circ$) were heated with 30 cc. of pyridine until solution was completed. To the pyridine solution 250 cc. of 98.5 per cent ethyl alcohol were added and the solution was allowed to stand at room temperature for about 3 hours. The solution turned into a solid mass. The first yield of α -mannose was about 60 per cent. The mother liquors were generally concentrated under diminished pressure and taken up again in hot pyridine. To the solution 98.5 per cent alcohol was added. On repeating the operation, α -mannose can be converted into the α isomer practically without loss. The rotation of the sample reported in this experiment in aqueous solution was

$$[\alpha]_D^{20} = \frac{+0.90^\circ \times 100}{1 \times 3} = +30^\circ$$

In some experiments, the material had a specific rotation of $[\alpha]_D^{20} = +28^\circ$.

Preparation of α -Mannose by Glacial Acetic Acid Process.—50.0 gm. of α -mannose ($[\alpha]_D^{20} = -11^\circ$) were dissolved in 20 cc. of hot water and the solution was cooled to 0°C . 100 cc. of cold glacial acetic acid (cooled until it began to crystallize) were added to the solution. On standing, the solution turned into a solid mass. The yield was 25.0 gm. and the specific rotation was

$$[\alpha]_D^{20} = \frac{+0.87^\circ \times 100}{1 \times 3} = +29^\circ$$

Also in these experiments, the mannose remaining in the mother liquors was easily recovered.

Preparation of α -Mannose by the Ammonia Process.—10 gm. of β -mannose were dissolved in 5 cc. of concentrated aqueous ammonia. To the solution, 100 cc. of absolute alcohol and then ether were added as long as an oil continued to settle out. The oil was taken up in small portions of dry methyl alcohol until it

crystallized. The dry substance had the following specific rotation.

$$[\alpha]_D^{20} = \frac{+0.72^\circ \times 100}{1 \times 3} = +24^\circ$$

Also, in this process the mannose remaining in the mother liquor could be completely recovered.

Purification of α -Mannose.—For purification, the crude material was allowed to stand for 24 hours in double its weight of 80 per cent alcohol. The residue was shaken for 5 minutes with a small portion of 80 per cent alcohol at 20°C . To a portion of the filtrate a few drops of ammonia were added and the optical rotation was measured. The extraction was continued until the rotation remained constant for three subsequent extractions. The rotation of these extracts in a 1 dm. tube was $\alpha = +2.30^\circ$. The residue was then washed with absolute alcohol and ether and dried under diminished pressure at 50°C . The substance melted into a semisolid mass at 133°C . (corrected) and turned liquid and decomposed at 205°C . It had a specific rotation in water of $[\alpha]_D^{20} = +30^\circ$, and in 80 per cent alcohol of $+35^\circ$.

Initial and Final Solubilities of α -Mannose in 80 Per Cent Alcohol.—The experiment was carried out at 20°C . 10.0 gm. of the dry α form were shaken in a glass stoppered flask for 5 minutes with 40 cc. of the solvent. About 10 cc. of the supernatant liquid were withdrawn by a Lowry pipette, 2 drops of ammonia water were added to the solution, and the rotation was measured. $[\alpha]_D$ was $+2.30^\circ$. The flask with the remainder of the suspension was allowed to stand for 24 hours when another sample of 10 per cent was withdrawn. The rotation was $+2.80^\circ$.

The rotation of the β form is calculated from the equation $0.821(35) + 0.179(x) = 25.7$. 25.7 is the equilibrium specific rotation of mannose in 80 per cent alcohol. x (the rotation of the β form) is $+16.5^\circ$.

Initial and Final Solubilities of β -Mannose in 80 Per Cent Alcohol.—The experiment was carried out in the same manner as in the case of α -mannose. 10.0 gm. of β -mannose were suspended in 75 cc. of 80 per cent alcohol. The rotation of the first extract was $+0.38^\circ$, of the final $+1.80^\circ$. The specific rotation of the α form, calculated from the equation $0.188(-16.5) + 0.812(x) = 25.7$, is $+35^\circ$.

The Solubility of a Mixture of α - and β -Mannoses.—The β -mannose was purified in the same way as the α isomer. It melted into a semisolid mass at 140°C . (corrected), and then behaved as the α form. A concentrated solution in 80 per cent alcohol in equilibrium gave an optical rotation of $+0.38^{\circ}$ in a 100 mm. tube.

A mixture of 3 gm. of β -mannose and of 7.0 gm. of α -mannose was shaken for 5 minutes at 20°C . with 30 cc. of 80 per cent alcohol. The filtrate, after addition of a drop of ammonia, had the optical rotation of $[\alpha]_D^{20} = +2.67^{\circ}$ in a 100 mm. tube. The calculated value is $+2.68^{\circ}$.

Mutarotation of α - and β -Mannoses at 25°C .—5 gm. of dry α -mannose were shaken in a thermostatic bath at 25°C . for 5 minutes. The supernatant liquor was rapidly filtered. 3 gm. of the β form were treated in the same way. The measurements were taken in tubes provided with jackets through which circulated the water from the same thermostatic bath. The changes of rotation were as follows:

Time.	α -Form.	$k_1 + k_2$	Time.	β -Form.	$k_1 + k_2$
<i>min.</i>			<i>min.</i>		
0	+6.95		0	-0.72	
27	+6.25	0.00775	30	-0.07	0.00545
57	+5.79	0.00795	62	+0.43	0.00572
89	+5.50	0.00770	91	+0.73	0.00580
116	+5.31	0.00850	120	+0.95	0.00602
Equilib- rium.	+5.12	Average 0.00792	Equilib- rium.	+0.34	Average 0.00549

Mutarotation of α - and β -Mannoses at 12.5°C .—These measurements were carried out in a room in which the temperature was kept constant at 12.5°C . 0.500 gm. of dry β -mannose was dissolved in 25 cc. of 80 per cent alcohol. It took about $2\frac{1}{2}$ minutes to complete solution. 3 gm. of α -mannose were shaken 5 minutes with 3 cc. of 80 per cent alcohol. Under these conditions, the concentrations of the α - and β -mannoses were approximately the same.

The course of the mutarotation was as follows:

Time.	α -Form.	$k_1 + k_2$	Time.	β -Form.	$k_1 + k_2$
<i>min.</i>			<i>min.</i>		
0	+1.33		0	-0.66	
34	+1.26	0.00219	30	-0.57	0.00169
76	+1.19	0.00239	59	-0.37	0.00144
113	+1.11	0.00247	97	-0.22	0.00141
147	+1.11	0.00255	121	-0.11	0.00148
Equilib- rium.	+0.95	Average 0.00240	Equilib- rium.	+0.97	Average 0.00150

Two other experiments were performed. The respective values were 0.00241 and 0.00239 for the α , and 0.00170 and 0.00141 for the β form. Recalculated for 25°C. by the coefficient of Hudson and Sawyer (2.6 for 10°) the values given in the table become 0.00753 for the α form and 0.00567 for the β form. These values agree very closely with those found in the experiments reported above.

Preparation of β -Glucose.—200 gm. of α -glucose were dissolved in about 50 cc. of hot water; to this solution, 20 cc. of concentrated ammonia water were added. The solution was allowed to stand 5 minutes. Then 200 cc. of 98.5 per cent alcohol were added. To the resulting solution ether was added as long as an oil settled out. The supernatant liquid was then decanted and to the syrup methyl alcohol was added in small portions, while stirring and scratching on the walls of the vessel were continued. The glucose crystallized immediately. The yield was 140 gm. The specific rotation of the crude material was $[\alpha]_D^{20} = +23^\circ$. The material remaining in the mother liquor was easily recovered.

Preparation of β -Galactose.—50 gm. of α -galactose were dissolved in 40 cc. of concentrated ammonia water in the cold. 200 cc. of cold alcohol were added and then ether was added as long as oil settled out. The supernatant liquid was decanted, and to the oil small portions of methyl alcohol were added, while stirring and scratching on the walls of the vessel were continued. The crystallization began almost at once. The specifications of the crude β form were $[\alpha]_D^{20} = +63^\circ$.

THE TWO ISOMERIC CHONDROSAMINE HYDROCHLORIDES AND THE RATES OF THEIR MUTAROTATION.

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The first sample of chondrosamine hydrochloride isolated by Levene and La Forge¹ had an initial rotation of $[\alpha]_D^{20} = +129^\circ$, which reached an equilibrium at the rotation of $[\alpha]_D^{20} = +95^\circ$. All subsequent preparations, both synthetic and natural, had an initial specific rotation in the neighborhood of $[\alpha]_D^{20} = +51-60^\circ$ and an equilibrium rotation of $[\alpha]_D^{20} = +95^\circ$. Since chondrosamine belongs to the *d* series, the second form is to be regarded as the β isomer. In this respect, chondrosamine represents one of the few sugars which, under normal conditions, crystallizes in the β form. The common form of most other simple sugars, with the exception of mannose, is the α form.

The knowledge of the rotation of the α and β isomers is important for the purpose of obtaining information as to the value and the direction of the rotation of carbon atom 2 in the sugars. The bearing of the direction of carbon atom 2 on the configuration of sugars and sugar acids has been discussed in the paper on epiglucosamine pentacetate.² In view of this, an effort was made to prepare both isomers of chondrosamine hydrochloride in pure form. Since the prevailing material consists of the β isomer, the first step was to obtain the equilibrium form. A convenient way to prepare this form is given in the experimental part. This material was extracted repeatedly either with methyl alcohol or with a solution consisting of equal parts of absolute ethyl and methyl alcohols. By the addition of ether to the filtrate, a substance was obtained which had a higher rotation than the initial substance. On repeating the operation, the rotation of the more

¹ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 127.

² Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1923, lv, 221.

soluble form gradually increased and finally reached the value $[\alpha]_D^{20} = +121^\circ$.

On the other hand, a method was found to purify the β isomer so that its rotation came down to $[\alpha]_D^{20} = +47^\circ$. The extrapolated initial rotation was found to be $[\alpha]_D^{20} = +44.5^\circ$. Thus, the difference of the molecular rotation of the two forms is 16,485. It is possible that the α form was not yet absolutely pure and that the pure substance possesses a slightly higher rotation than $+121^\circ$, but the difference of the molecular rotations of the two forms is practically identical with the normal value for the majority of sugars. It is also very close to the value obtained by Irvine and Earl for the difference of the rotation of the two isomeric chitosamine hydrochlorides. The fact that the mutarotations of the α and β forms gave the same value for $k_1 + k_2$ speaks in favor of the purity of the two substances. The discrepancy in the rotation of the sample of Levene and La Forge and of the present samples may in part be due to the difference in the details of the technique of measurement. Whereas, in previous work the measurement was carried out in closed tubes, the present measurements were made in tubulated tubes.

EXPERIMENTAL PART.

Purification of Chondrosamine Hydrochloride.—3.0 gm. of crude material with a specific rotation of $[\alpha]_D^{20} = +80^\circ$ were dissolved with heat in 80 cc. of concentrated hydrochloric acid. An additional 7.0 gm. of substance was added to the solution in small portions. To this solution alcohol was added until a crystalline deposit formed. The precipitate was filtered off while the mixture was still warm. This substance melted with decomposition at 187°C . 3 minutes after the substance was mixed with the solvent the rotation was

$$[\alpha]_D^{25} = \frac{+0.96^\circ \times 100}{1 \times 2} = +48^\circ$$

The mother liquors of this material were concentrated nearly to dryness and dissolved in a minimum amount of hot concentrated hydrochloric acid. Absolute alcohol was added until the substance began to crystallize. The optical rotation of this substance 4 minutes after solution was

$$[\alpha]_D^{25} = \frac{+1.38^\circ \times 100}{1 \times 3} = +46^\circ$$

The rate of mutarotation of the β form was as follows:

Time.	α	$k_1 + k_2 = \frac{1}{t} \log \frac{r_\infty - r_0}{r_\infty - r_t}$
4 min.	+0.96	
15 "	+1.20	0.0154
27 "	+1.40	0.0131
37 "	+1.49	0.0121
47 "	+1.57	0.0121
133 "	+1.84	Average.....0.0131
24 hrs.	+1.84	

On the basis of these values the extrapolated initial specific rotation was $[\alpha]_0^{20} = +44.5^\circ$.

Preparation of α -Chondrosamine Hydrochloride.—50 gm. of the substance with specific rotation $[\alpha]_D^{15} = +0.56^\circ$ were dissolved in 600 cc. of warm 60 per cent alcohol. The solution was allowed to remain on the water bath for half an hour when 600 cc. of acetone were added. An oily sediment formed. Methyl alcohol was then added until the sediment acquired a crystalline character and then 400 cc. of acetone were gradually added with stirring. The mixture was allowed to stand for 1 hour and then filtered. The molecular rotation of this material was $[\alpha]_D^{25} = +67.5^\circ$. The filtrate was concentrated to a syrup under diminished pressure at 49°C . (water bath temperature). The syrup was dissolved in a little methyl alcohol. To the solution acetone was gradually added as long as its addition produced a cloudiness. The crystalline precipitate was filtered off after 15 minutes. Its specific rotation was $[\alpha]_D^{20} = +95^\circ$. This value is that of the equilibrium form.

The pure α isomer was prepared by extracting the equilibrium form with methyl alcohol or with a mixture of ethyl and methyl alcohol. 20 gm. of the dry material were extracted at 15°C . for 3 minutes with 150 cc. of dry methyl alcohol and filtered. To the filtrate ether was added as long as it produced a turbidity. The crystalline precipitate was filtered off immediately. The extraction was repeated twice and the three crystalline deposits obtained from the filtrates by the addition of ether, were combined. The yield was 5.0 gm. and the substance had the specific rotation of $[\alpha]_D^{20} = +121^\circ$. On further purification, the rotation of the

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substance did not change. The final substance had a melting point of 185 C. and analyzed as follows:

0.1974 gm. substance: (Kjeldahl) 9.30 cc. 0.1 N acid.

0.1974 " " : (Volhard) 9.10 " 0.1 " AgNO₃.

0.1072 " " : 0.1306 gm. CO₂ and 0.0630 gm. H₂O.

C₆H₁₂NO₃HCl. Calculated. C 33.40, H 6.54, N 6.54, Cl 16.45.

Found. " 33.22, " 6.59, " 6.59, " 16.31.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+2.42^\circ \times 100}{1 \times 2} = +121^\circ$$

In other experiments in which the mixture of the α and β forms had a specific rotation lower than $+90^\circ$, the α isomer was obtained more successfully by extractions with a mixture of methyl and ethyl alcohol.

The rate of mutarotation of the substance was as follows. 7 minutes were required to dissolve the substance, filter the solution, and take the first reading:

Time.	α	$k_1 + k_2 = \frac{1}{t} \log \frac{r_\infty - r_0}{r_\infty - r_t}$
<i>min.</i>		
7	+2.36	
11	+2.21	0.0155
31	+2.07	0.0133
59	+1.96	0.0139
76	+1.90	0.0134
Equilibrium.	+1.86	Average.....0.0140

ON THE IDENTITY OR NON-IDENTITY OF ANTINEURITIC AND WATER-SOLUBLE B VITAMINS.

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The problem to be reported in this paper has been the subject of much discussion in recent literature. A thorough analysis of the evidence on both sides of the questions was presented first by Mitchel and later by Sherman and Smith. The very impartial scrutiny of all the evidence leads these authors to the conclusion that the evidence to date does not permit of a final decision in either direction. The present communication was made with a view of presenting evidence on the non-identity of the two vitamins. The observations were incidental in an effort to organize the work on the chemical nature of the water-soluble B vitamin. The original aim was to select a convenient source of vitamin B and a convenient laboratory animal. As the source of vitamin, various samples of yeast were tested and as laboratory animals, the pigeon and the white rat (descendants of Osborne and Mendel's colony, obtained through the courtesy of Prof. H. Sherman) were selected. It was then incidentally observed that certain samples of yeast which were sufficiently active to maintain normal growth in rats failed to protect pigeons fed on polished rice from polyneuritis. This observation led to a more careful investigation into the present problem. Three samples of bakers' yeast supplied by the Fleischmann Company, and one sample of brewers' yeast were employed in the investigation.

The results are summarized in the tables and chart, from which it is seen that the water-soluble B vitamin of four samples of yeast varied comparatively little. In daily doses of 0.200 gm., all the samples were effective. In 0.100 gm. doses, brewers' yeast and bakers' yeast No. 3 were sufficient to maintain normal growth, whereas Nos. 2 and 9 in such doses gave less satisfactory results. Thus, the proportional difference between the best and the poorest samples was not greater than 2 to 1.

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The results of feeding pigeons with the same samples of yeast were the following.

Brewers' yeast protected pigeons from polyneuritis in doses of 0.500 gm. per day; bakers' yeast No. 3 gave irregular results in doses of 0.200 gm. per day and protected completely in doses of 0.500 gm. per day. Bakers' yeast No. 9 failed to protect in doses of 0.500 gm. and gave irregular results in doses of 1.0 gm. (of three pigeons, one lived 50 days and two remained in perfectly normal condition after 70 days). Bakers' yeast No. 2 failed to protect even in doses of 2.0 gm. per day. Thus, No. 2 failed to protect pigeons from polyneuritis in doses at least four times as large as the protective dose of No. 3.

Occasionally, in a few earlier experiments, it seemed that an extract of a certain sample of yeast contained the protective power when the untreated yeast failed to do so. The extract was prepared following the directions of Osborne and Wakeman. A more careful investigation, however, revealed that the impression was erroneous. The Osborne and Wakeman fraction II obtained from yeast No. 2 was inactive in doses of 0.500 gm., whereas a similar fraction from the brewers' yeast carried a certain degree of protection in doses of 0.075 gm. per day and completely protected in doses of 0.125 gm.

It is noteworthy that whereas the concentration of the protective principle was higher in yeast No. 3 than in the brewers' yeast, the concentrations of the principle in the Osborne and Wakeman fraction II were reversed, being lower in that from yeast No. 3 than from the brewers' yeast. All samples of the Osborne and Wakeman fraction II in daily doses of 0.020 to 0.050 gm. were effective in maintaining the normal growth of rats fed on the basal diet of Osborne and Mendel.

All postmortem examinations were made by Dr. P. Olitsky. Only animals sacrificed before death or found dead during laboratory hours were autopsied.

Thus, the material presented in this report contains additional evidence in support of the view that the antineuritic and the growth-promoting principle are not identical. However, it must be borne in mind that the final solution of the problem will be furnished only by the knowledge of the chemical nature of the active principles.

EXPERIMENTAL.

In the rat experiments, the technique of Osborne and Mendel was followed throughout. Young rats were placed in individual cages on the basal vitamin B-free diet until they had definitely lost weight, when vitamin in tablet form was given. Careful records of food consumption were kept and since the cages had wire mesh bottoms, there was little or no contamination or eating of feces.¹ All the tests were made on a series of four or more rats. The curves published are typical of the series.

The vitamin B-free basal diet employed was

	<i>per cent</i>
Casein.....	18
Salt mixture IV.....	4
Starch.....	54
Butter fat.....	9
Lard.....	13
Cod liver oil.....	2

For the pigeon experiments, birds weighing over 300 gm. were used. They were kept in individual cages and were forcibly fed 20 gm. of ground white rice, 18 cc. of distilled water, and the vitamin daily. All experiments were prophylactic and were continued at least 63 days on a series of three birds. Controls on 20 gm. of ground mixed grains were found healthy and normal.

¹ Our cages were made of galvanized iron, 9 inches in diameter. They were made in three pieces; the wire mesh base with a rim to hold the cage, and a cover weighted with a lead rim. The cage stood in a deep agate pan over a layer of sand, which may be moistened with 2 per cent sulfuric acid in order to remove effectively any odor.

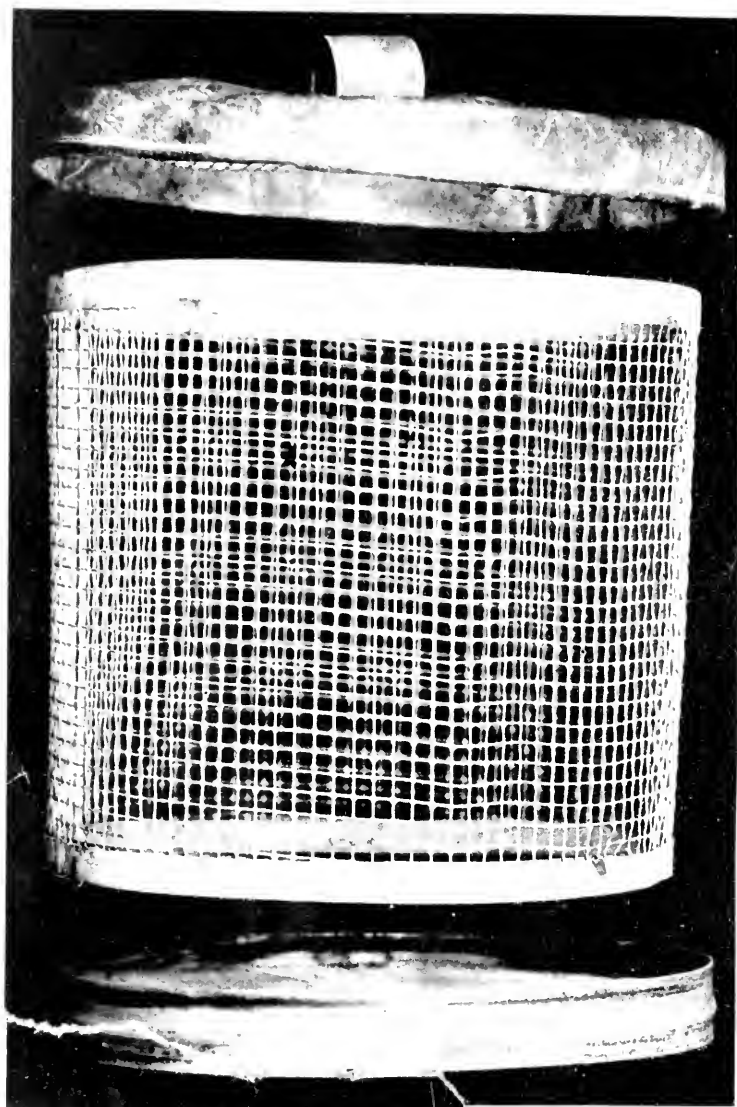


FIG. 1.

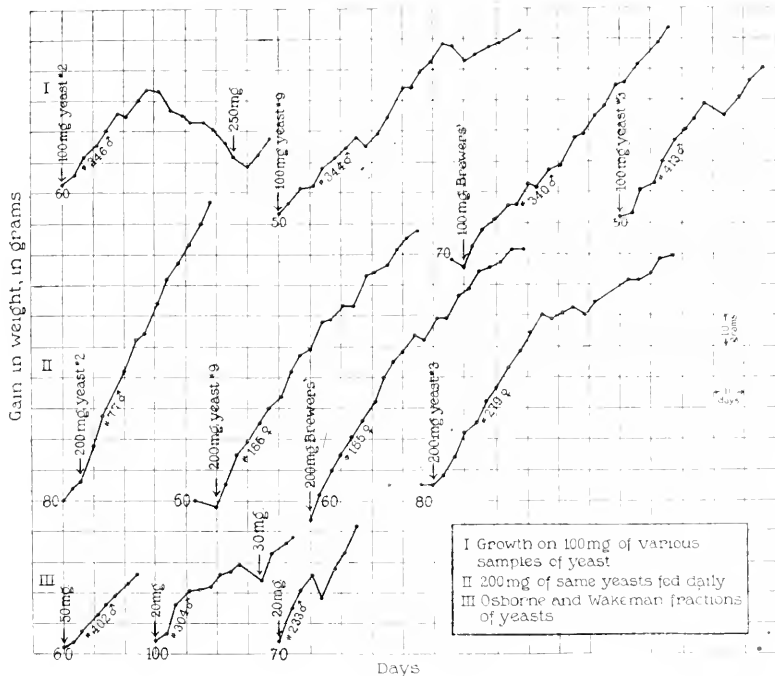


CHART 1.

TABLE I.
Bakers' Yeast No. 2 on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
		<i>days</i>	<i>gm.</i>	
200 mg.	17	32	+2	Spastic; killed.
	18	20	+4	Died.
	19	22	-32	Spastic; died.
	20	20	0	" "
500 mg.	74	21	-50	Convulsions.
	75	14	-7	"
	76	17	+10	Crop full; spastic.
1 gm.	92	17	Crop full.	Convulsions. Autopsy; polyneuritis.
	93	33	-106	Died.
	94	30	+70	"
2 gm.	177	77	-10	Autopsy; normal.
	179	77		
	180	68	-55	Autopsy; polyneuritis.

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TABLE II.
Osborne and Wakeman's Fraction II from Bakers' Yeast No. 2.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
<i>mg.</i>		<i>days</i>	<i>gm.</i>	
75	148	21	-35	Autopsy; polyneuritis.
	149	25	Crop full.	" "
	150	18	-27	Spastic.
125	151	28	-77	Autopsy; polyneuritis.
	152	28	-35	Died.
	153	21	-43	Autopsy; polyneuritis.
150	181	21	-10	Autopsy; almost normal.
	182	26	-41	" polyneuritis.
	183	26	Crop full.	Paralysis. Autopsy; polyneuritis.
175	154	27	-44	Spastic.
	155	28	Crop full.	Died.
	156	21	-23	Autopsy; polyneuritis.
250	184	26	-42	Spastic. Autopsy; polyneuritis.
	185	23	-60	
	186	21	-18	Crop full. Autopsy; beginning polyneuritis.
500	187	42	+8	Crop full; died.
	188	49	+40	Autopsy; polyneuritis.
	189	56	-25	" "

TABLE III.
Osborne and Wakeman's Fraction II from Bakers' Yeast No. 3.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
<i>mg.</i>		<i>days</i>	<i>gm.</i>	
75	157	35		Died.
	158	49	-47	Spastic; died.
	159	70	-18	Autopsy; mild polyneuritis.
125	161	70	-39	Well.
	162	70	-30	"
175	163	70	0	"
	164	39	-30	Autopsy; beginning polyneuritis.
	165	70	-30	Well.

TABLE IV.

Bakers' Yeast No. 3 on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
		<i>days</i>	<i>gm.</i>	
200 mg.	101	70	-8	Apparently healthy.
	102	70	-20	Autopsy; normal.
	103	53	-72	" polyneuritis.
500 mg.	104	70	-35	Well.
	105	70	-11	"
	106	70	+64	"
1 gm.	107	70	-8	"
	108	70	+42	"
	109	70	+4	"

TABLE V.

Bakers' Yeast No. 9 on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
		<i>days</i>	<i>gm.</i>	
200 mg.	54	21	-34	Spastic; died.
	55	40	0	Crop full; died.
	56	35	-10	Died suddenly.
	57	34	+9	Paralyzed; died.
500 mg.	80	28	-10	Atropic; died.
	81	28	-2	Spastic; died.
	82	25	+28	Crop full; died.
1 gm.	97	49	+5	Autopsy; beginning polyneuritis.
	98	77	+17	" normal.
	99	70	+15	Healthy.

TABLE VI.

Osborne and Wakeman's Fraction II from Bakers' Yeast No. 9.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
<i>mg.</i>		<i>days</i>	<i>gm.</i>	
75	139	23	-10	Autopsy; polyneuritis.
	140	49	-94	Weak; returned to normal on 200 mg.
	141	25	-60	Autopsy; polyneuritis.
125	142	70	-80	" very beginning of polyneuritis.
	143	70	-12	
	144	33	-49	Autopsy; mild polyneuritis.
175	145	42	-31	Died.
	146	28	-85	Autopsy; polyneuritis.
	147	70	+6	Apparently healthy.

TABLE VII.

Brewers' Yeast on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
		<i>days</i>	<i>gm.</i>	
200 mg.	67	34	Crop full.	Convulsions; died.
	69	30	-25	" "
	70	20	+45	Crop full. Autopsy; polyneuritis.
500 mg.	86	63	+36	Well.
	87	63	-13	"
	88	63	-18	"
1 gm.	89	70	+1	"
	90	70	-8	"
	91	70	+16	"

TABLE VIII.

Osborne and Wakeman's Fraction II from Brewer's Yeast.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
<i>mg.</i>		<i>days</i>	<i>gm.</i>	
75	130	65	-29	Autopsy; normal.
	131	65	-2	" polyneuritis.
	132	35	-43	Died.
125	133	63	+8	Autopsy; normal.
	134	63	+25	" "
	135	63	+25	" "
175	136	65	+3	" "
	137	65	-8	" "
	138	65	-25	" "

A STUDY OF THE INORGANIC ELEMENTS OF BLOOD PLASMA.

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A number of papers during the past 10 years have reported variations in the concentration of calcium or inorganic phosphorus of the blood, in rickets, nephritis, and in parathyroidectomized animals. Reports have appeared also concerning changes in the level of other inorganic constituents of the blood in a variety of diseases. But in none of this work has more than a few of the inorganic elements been determined in the same bloods. For the purpose of obtaining a more comprehensive view of the extent of variations in the inorganic constituents we have developed a system of analysis which permits the determination of sodium, potassium, calcium, magnesium, chlorides, and phosphates on a single small sample of blood. The bloods of fifty different patients, selected to include a variety of diseases, have been analyzed for these constituents, and the results may serve to indicate the extent of variations to be found in the more frequent pathological conditions.

Methods.

Most recent work on the inorganic elements of the blood has been done on whole blood or serum, and results on serum seem to have more significance. Since plasma can be obtained in larger amounts than serum and carries the same significance, methods have been adapted to this fraction of the whole blood.

Anticoagulant.—When all of the elements are to be determined on the same specimen, the choice of anticoagulant becomes important. Lithium citrate seems to fill all the requirements. It may be obtained fairly pure on the market, or purified if necessary by precipitating a saturated solution with an equal volume

of 95 per cent alcohol. It effectively prevents clotting, and as shown by results in Table I, it does not affect the analytical results.

Blood is drawn from superficial veins of the forearm and transferred to a 50 cc. centrifuge tube containing about 3 mg. of lithium citrate per cubic centimeter of blood; after centrifugation at high speed for a few minutes the plasma is drawn off for analysis. From 9 to 15 cc. of plasma, the amount required for a complete analysis, are measured by a Folin-Wu pipette and transferred to

TABLE I.
Effect of Lithium Citrate (3 Mg. per Cc.).

	Beef serum.	Beef serum + lithium citrate.	Defibrinated beef blood.	Beef blood + lithium citrate.
P.....	7.1	7.1		
Ca.....	12.4	12.6	7.8	7.8
Mg.....	2.7	2.4	3.8	3.7
K.....	17.9	18.1	64.6	63.5
Na.....	322	325	280	284
NaCl.....	597	603	556	556

an Erlenmeyer flask, diluted with 3 volumes of distilled water, and the proteins precipitated with an equal volume of 20 per cent trichloroacetic acid. After shaking vigorously for a minute and standing 10 minutes the contents are poured onto a dry filter.

Preparation of Filters.—Ordinary filters contain various salts and ashless filters contain hydrochloric acid so that any filters used will require preliminary washing and drying. The following treatment has been found satisfactory. A pack of ordinary 11 cm. filters is treated in a large dish with dilute nitric acid for about 1 hour, and then washed repeatedly by decantation with distilled water, and dried.

Analytical Procedure.—The method for the determination of potassium, which is new, will be given in detail, the other methods are adaptations of well known procedures and may be given only by reference.

Potassium Method.—Use is made of the nitrite color reaction of Griess, to determine the nitrite precipitated with potassium as potassium cobalti-nitrite. This method was developed by Doisy

and Bell, but has not hitherto been published. It is described here, with a few alterations by the present writer, with their consent.

Transfer 5 cc. of the trichloroacetic acid filtrate to a small glass evaporating dish or beaker. Add 3 or 4 drops of concentrated HNO_3 and evaporate to dryness on the steam bath. Dissolve the residue in 1 cc. of distilled water, add 2 cc. of the cobalti-nitrite reagent, and set aside 4 hours for precipitation. Filter onto a Gooch crucible prepared as follows: Pour into the Gooch enough paper pulp to make a mat about 1 mm. thick, cover with a piece of filter paper punched out with a cork borer to fit, and press the whole mat down flat and tight. Wash out the beaker four times with 3 cc. portions of 10 per cent NaCl and pass these through the Gooch, then wash the crucible twice with about 3 cc. of the same solution. Pick out the mat and drop it back in the dish with the rest of the precipitate, add about 20 cc. of approximately $0.1 \times \text{NaOH}$, and heat to boiling. Wash the contents into a graduated cylinder, dilute with distilled water to 100 cc., and mix. An aliquot of this solution equivalent to about 0.01 mg. of K (5 cc. where 5 cc. of trichloroacetic acid filtrate were used) is transferred to a 100 cc. volumetric flask. To another 100 cc. flask transfer 5 cc. of the standard nitrite solution. To each add 70 cc. of distilled water, 2 cc. of the sulfanilic acid solution, and 1 cc. of the naphthylamine solution. Dilute to 100 cc., mix, and after 5 minutes compare in the colorimeter.

Reagents.

Potassium Solution.—This contains the equivalent of 20 mg. of K per 100 cc. 382 mg. of pure KCl or 445 mg. of K_2SO_4 are dissolved in a liter of distilled water.

Cobalti-Nitrite Reagent.—Dissolve 220 gm. of NaNO_2 in 400 cc. of distilled water. Dissolve 113 gm. of cobalt acetate in 300 cc. of distilled water. Mix the two solutions and add 100 cc. of glacial acetic acid. A current of air is passed through the reagent for several hours to remove the nitric fumes. It is then filtered and kept in a glass stoppered bottle in the ice box.

Standard Nitrite Solution.—Prepare a stock solution by dissolving 1.5 gm. of KNO_2 in 1,000 cc. of distilled water. The standard solution is made by diluting 10 cc. of the stock solution to 1,000 cc. It is standardized empirically as follows: Transfer 2 cc. of the potassium solution, equivalent to 0.4 mg. of K to a small beaker, precipitate, wash, decompose with NaOH , and make up to a volume of 200 cc. as in a determination. Take 5 cc. of this solution equivalent to 0.01 mg. of K and compare according to the technique of the determination with 5 cc. of the standard nitrite solution. The value of the nitrite standard is thus established in terms of its potassium equivalent.

Sulfanilic Acid Solution.—A 0.5 per cent solution in 30 per cent acetic acid.

α -Naphthylamine.—A 0.5 per cent solution in 30 per cent acetic acid.

Remarks.—The mat of paper pulp is much more efficient than asbestos for catching finely divided precipitates such as potassium cobalti-nitrite. Reducing substances which are formed by the action of NaOH on the paper during decomposition of the cobalti-nitrite and which would introduce an error if the nitrite were determined by permanganate titration, have no effect on the colorimetric method.

The precipitate was originally caught on a filter paper and washed with 10 per cent NaCl which flocculates the precipitate and prevents it from running through the filter. Using the Gooch, much smaller amounts of wash fluid are required and the pulp mat makes a better filter so that there would probably be no appreciable loss if distilled water were used for washing.

Sodium is determined by the method of Kramer and Tisdall (1). From 5 to 10 cc. of the trichloroacetic acid filtrate are evaporated with 5 cc. of concentrated HNO_3 to dryness on a steam bath. The residue is dissolved in 2 cc. of distilled water and the procedure given by Kramer and Tisdall followed from this point.

Determinations made directly on the trichloroacetic acid filtrate gave results much too low, perhaps due to interference by the citrate. All interference is removed by evaporation with HNO_3 and with this treatment results are identical with and without added lithium citrate.

Glass evaporating dishes of 50 cc. capacity have been used and although the precipitate has a strong tendency to stick to the glass this may be overcome by rubbing the sides vigorously with a rubber policeman during and for a few seconds following the addition of the alcohol.

The necessity of preparing an antimonate reagent, 10 cc. of which are equivalent to about 11 mg. of sodium, should be especially emphasized. Different samples of antimonate have been found to vary greatly in their antimony content and, with one exception, solutions which gave results higher than theoretical have been obtained by dissolving 10 gm. of the antimonate in 500 cc. of boiling water, etc., as directed by Kramer and Tisdall (1).

Calcium and Magnesium.—15 cc. of the trichloroacetic acid filtrate are used for these two determinations. Calcium is precipitated as oxalate, after neutralizing the filtrate with dilute ammonia, using methyl red for an indicator as recommended by Shohl (2). The precipitate was filtered, washed, and titrated somewhat according to the technique of Simpson (3).

Sodium or potassium acetate solutions should not be used to neutralize the trichloroacetic acid filtrates because the large amounts required to reduce the acidity to the turning point of methyl red, retard the precipitation so that it will not be complete even after 10 hours standing.

Magnesium is determined on the calcium filtrate according to a procedure recently published from this laboratory (4).

Phosphates are determined by a modification of the Bell-Doisy method (5).

The method of Whitehorn (6) has been adapted to the trichloroacetic acid filtrates for the determination of chlorides.

Inorganic Elements in Various Pathological Conditions.

In Table II are given analyses of fifty different pathological plasmas. Several of the nephritics show the characteristic low values for calcium or high values for phosphates. Aside from these it will be observed that there are no conspicuous deviations from the normal for any of the elements in any of the bloods examined.

Leaving out the results on nephritis, the following averages are obtained.

P	Cl	Na	K	Ca	Mg
2.9	355	317	19.6'	9.6	2.5

These values are very close to those which have been cited (7, 8, 9) as average normal for human serum or plasma.

A comparison of the equivalent concentrations of the acid and basic elements, based upon the average results, are given in Table III. A concentration of 0.0245 N has been assumed for HCO_3 , corresponding to a value of 55 cc. of CO_2 per 100 cc. of plasma. The value of 0.0014 N for phosphoric acid has been computed, assuming that it exists in equivalent amounts as mono-basic and di-basic phosphate. No figure for sulfuric acid has been included, since the recent work of Denis (10) shows this element to exist only in very small amounts except in conditions of renal impairment. There is seen to be an excess of about 16 per cent of the basic over the acid elements, which compares well with the 14 per cent excess computed by Kramer and Tisdall (8) for normal human serum and is a little higher than 7 per cent computed by Doisy and Eaton (7) for normal beef serum. The excess base no doubt exists as alkali proteinate (8).

TABLE 11.

Patient No.	Diagnosis.	P	Cl	Na	K	Ca	Mg
1	Aene.	2.6	364	329	18.0	10.0	2.2
2	"	3.6	349	296	18.1	9.8	2.3
3	"	3.5	359	316	21.6	10.1	2.4
4	Psoriasis.	2.9	370	309	19.0	10.0	2.9
5	Urticaria.	2.2	340	334	17.7	8.7	3.1
6	Arthritis (infective).	3.6	360	338	16.9	9.7	2.4
7	Pulmonary tuberculosis.	2.5	334	306	19.0	11.2	2.6
8	"	2.1	370	339	18.8	9.1	2.4
9	Asthma.	2.9	355	303	17.5	11.5	2.5
10	"	3.1	348	318	20.0	9.3	3.3
11	Bronchial pneumonia.	1.2	338	315	19.7	9.3	2.2
12	Eclampsia.	3.8	372	349	20.9	8.8	2.3
13	"	2.8	349	308	16.3	8.9	2.0
14	Carcinoma.	2.7	342	294	15.9	9.5	2.5
15	"	3.4	371	339	20.6	9.8	2.6
16	"	3.1	334		19.3	9.5	2.9
17	"	3.6	349	316	22.9	9.7	2.6
18	Sarcoma.	2.8	364	328	17.8	9.6	2.8
19	Myeloma.	4.0	340	311	23.4	10.0	2.5
20	Polycythemia.	3.2	364	317	20.0	10.1	2.5
21	"	2.8	343	288	22.9	8.4	
22	Pernicious anemia.	3.6	373	340	20.0	9.4	2.6
23	"	3.0	349	302	16.8	9.1	2.5
24	Hemophilia.	2.3	369	336	17.1	10.1	2.5
25	Tetany (hysterical hyperpnea).	3.7	354	323	19.2	10.1	2.9
26	Addison's disease.	2.8	355	306	21.6	10.0	2.2
27	Hypoovarian (castrated).	3.1	379	338	18.5	9.1	2.5
28	Acromegaly.	2.1	341	291	19.7	10.1	2.1
29	Diabetes mellitus.	3.7	352	334	23.3	9.5	2.3
30	Hyperthyroid.	1.6	369	321	20.0	9.7	2.8
31	"	2.0	358	314	24.5	9.7	2.3
32	"	3.1	371	337	18.3	10.1	2.5
33	Muscular dystrophy.	2.6	350		17.4	11.2	2.7
34	Chronic jaundice.	2.6	357	289	20.2	9.9	2.4
35	"	2.4	358	336	14.9	10.3	2.9
36	Cardiac decompensation.	3.4	355	317	21.3	9.6	2.2
37	"	3.2	335	283	24.1	9.4	2.6
38	"	2.8	342	296	17.7	10.3	2.5
39	Hypertension.	2.8	353		19.9	9.6	3.2
40	"	2.0	369	315	21.4	10.0	2.7
	Chronic nephritis.	2.1	349		18.8	8.1	2.4
	"	4.5	342		17.6	7.8	3.2
	"	8.1	349	329	16.9	7.5	2.5
	"	3.6	401	380	18.8	9.9	2.3
	"	5.9	343	293	18.0	8.1	2.6
	"	2.3	342	279	20.4	8.5	2.3
	"	11.9	328	308	36.2	8.7	3.9
	"	5.2	371	294	24.2	8.7	2.8
	"	5.0	412	375	16.9	9.3	4.2

TABLE III.

	Amount per liter.	Equivalent normality.
Bases.		
	<i>gm.</i>	
Na.....	3.170	0.1380
K.....	0.196	0.0050
Ca.....	0.098	0.0049
Mg.....	0.025	0.0021
Total.....		0.1509
	0.1500	
	0.1259	
Excess base = 0.0241 or 16 per cent.		
Acids.		
Cl.....	3.550	0.1000
P.....	0.029	0.0014
HCO ₃		0.0245
Total.....		0.1259

SUMMARY AND CONCLUSION.

A system of methods is outlined, including a new method for potassium, for the determination of the inorganic elements in blood plasma.

Analyses of a number of pathological plasmas are presented which, except in nephritis, show fairly normal values.

In view of the limited number of plasmas analyzed it is hardly permissible to generalize, though it is clear that marked variations in the level of inorganic elements of blood are quite exceptional.

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CONCENTRATION OF INSULIN BY ADSORPTION ON BENZOIC ACID.

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In the preparation of insulin on a large scale, a problem which is of considerable importance is the concentration of dilute aqueous solutions of the potent material. On account of the expense and loss of potency which result in boiling down such solutions, it seemed desirable to investigate the possibilities of adsorption. It was found that the potent material could be almost completely removed from aqueous solutions by certain materials, such as decolorizing carbons, but attempts to redissolve the insulin from the solid by heating, altering the acidity, using a different solvent, etc., were only partially successful.¹ It was decided to try some reagent which could be used in a finely divided form, and which could be subsequently dissolved. For this purpose certain organic acids, such for example as benzoic and salicylic, suggested themselves on account of their relative insolubility in water and the fineness of division in which they come down when a dissolved salt is treated with an acid.

If a dilute solution of sodium benzoate in an impure aqueous solution of insulin is acidified, finely divided benzoic acid is precipitated and carries down with it a large percentage of potent material. The following is a detailed procedure for concentrating insulin by this method. To 1 liter of a crude aqueous extract 50 cc. of a 25 per cent sodium benzoate solution are added and the solution is acidified with 12.5 cc. of concentrated hydrochloric acid. This is usually sufficient to saturate the solution with benzoic acid and hence to give a lasting precipitate; these quan-

¹We have later worked out a successful method of removing insulin from carbon, the details of which will be reported shortly.

ties can be increased or diminished proportionately, depending on the amount necessary to give the first lasting precipitate. Then 40 cc. of 25 per cent sodium benzoate and 10 cc. of concentrated hydrochloric acid are added, and after allowing sufficient time for complete precipitation the solution is filtered. This precipitate usually contains about two-thirds of the potent material. The filtrate is again treated with 40 cc. of 25 per cent sodium benzoate and 10 cc. of concentrated hydrochloric acid for a second precipitation. This is filtered and the resulting filtrate reprecipitated if it still contains sufficient potent material. The benzoic precipitate containing the insulin may be washed on the filter paper with a saturated aqueous solution of benzoic acid. A small amount of potent material comes off in the wash water which may either be reprecipitated along with the mother liquor, or may be collected and reconcentrated separately.

If this method of concentration is carried out on a highly purified solution the amount adsorbed is not so large as in an impure solution. This is probably due to the relative size of the particles of benzoic acid; the benzoic acid from the impure solution giving more adsorbing surface than that from a more purified solution.

Further, it is to be noted that even in very acid solutions the potent material is carried down in part. For example, at pH 1 one precipitation of a solution containing 150 units of insulin carried down 40 units of potent material. This is well out of the isoelectric range for insulin.

The insulin may be recovered in aqueous solution from the benzoic precipitate by various methods. If the original solution is comparatively free from protein the benzoic precipitate may be treated directly with ether and water to form two layers in a separating funnel. The benzoic acid is largely in the ether layer, and the potent material in the water layer. This water layer is washed with ether to remove traces of benzoic acid and the resulting aqueous solution is then boiled under vacuum to remove dissolved ether. It was noted that traces of ether had a distinct opposing or retarding effect on the action of insulin *in vivo*. If the solution to be concentrated is grossly impure, then sufficient inert material will come down with benzoic acid to form a distinct jelly when the benzoic precipitate is ethered directly.

One procedure in this case is to make up the moist precipitate to 80 per cent with ethyl alcohol, which dissolves the insulin and the benzoic acid; certain materials are allowed to settle out in the ice chest and the solution is filtered. The filtrate is boiled down under vacuum and ethered out as before. Another method of dealing with a precipitate which cannot be ethered directly is to dissolve the precipitate in glacial acetic acid. This solution is filtered after standing in the ice chest and either the potent material is precipitated in solid form by adding sufficient ether, or sufficient ether and water are added so that two layers form; the water layer containing the insulin. Traces of acetic and benzoic acids are removed from the aqueous solution by washing with ether.

This method of precipitating insulin with benzoic acid is successfully used in solutions containing 5 units in each 30 cc., the resulting solutions readily contain 5 units per cc. in a highly purified form. This method has been used on a large scale on the concentrate from the original alcoholic extract of the gland.

CONCLUSIONS.

A new type of adsorbing reagent has been successfully used. Insulin is by this means readily concentrated and greatly purified.



STUDIES IN THE PHYSIOLOGY OF MUSCULAR EXERCISE.

V. OXYGEN RELATIONSHIPS IN THE ARTERIAL BLOOD.

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The preceding articles of this series have dealt with the changes in CO_2 and the reaction of the blood resulting from short periods of exercise. The concomitant variations in oxygen capacity, oxygen content, and the saturation of hemoglobin are the subjects of the present discussion.

The results of previous investigations are apparently contradictory. Geppert and Zuntz (1) divided the spinal cord of dogs in the thoracic region and tetanized their hind legs. Some of the experiments were indecisive, but in one animal whose blood was drawn before and during exercise the oxygen capacity, oxygen content, and saturation of hemoglobin increased. It was inferred that arterial oxygen tension also rose. Hastings (2) obtained similar results on all but one of his dogs which had been running on a treadmill for several hours. Contrary evidence is found in the observation of Barcroft and his associates (3). In this experiment, work was performed on a bicycle ergometer in an atmosphere of which the partial pressure of oxygen had been reduced in 1 week from a sea-level value of 160 to 84 mm., equivalent to an altitude of 18,000 feet. As a result of exercise under these conditions the calculated oxygen content and tension and the observed saturation of hemoglobin diminished. In Harrop's (4) convalescent patient who exercised to exhaustion for 15 minutes at sea-level, a fall in content in conjunction with a rise in capacity yielded a greatly decreased saturation of hemoglobin. The results obtained by these different observers may be found in Table I.

TABLE I.
Results of Previous Investigation on Oxygen Relationships in Arterial Blood Before and After Exercise.

Author.	Content.			Capacity.			Hb saturation.			Remarks.
	Before.	After.	Difference.	Before.	After.	Difference.	Before.	After.	Difference.	
Geppert and Zuntz.	12.19	16.22	+4.03	16.09	18.56	+2.47	76	87	+11	Tetani- zation of hind legs of dog.
Hastings.	19.3	20.6	+1.3	21.3	22.4	+1.1	90.6	92.0	+1.4	Average of eight experiments on dogs running on tread- mill.
Barcroft and co- workers.	17.7	16.8	-0.9		20.1		88.1	83.5	-4.6	J. B. exercising on ergometer under reduced oxygen ten- sion.
Harrop.	21.09	19.9	-1.19	22.01	22.41	+0.37	95.6	85.5	-10.1	Exhausting exercise of con- valescent patient.

EXPERIMENTAL.

The procedure employed in the conduct of the experiments was described in detail in Paper I of this series (5). Exercise consisted of short periods of vigorous work performed on a Krogh bicycle ergometer. The methods of analyses were those of Van Slyke and Stadie (6). The protocols of the observations may be found in Papers I (5), II (7), and III (8) of this series.

Oxygen Content.—Oxygen content was found for blood drawn before, and during or after exercise in twenty experiments. The determinations were made in all cases immediately after the blood was drawn. The results are given in Table II.

In every instance the oxygen content is higher during and after exertion than under resting conditions. The return to normal seems to be gradual. In H.E.H., April 21, 1922, 3 minutes after exercise, the content was 21.8 volumes per cent; 15 minutes after, 20.4 volumes per cent; and in $\frac{1}{2}$ hour, 19.9 volumes per cent. The same phenomenon is observed in D.C.M., in an experiment not included in the protocols. 3 minutes after exercise, his content was 23.4 and in 15 minutes 22.2 volumes per cent.

Oxygen Capacity and Saturation of Hemoglobin.—As our early interests lay chiefly in the CO_2 capacity and the reaction of the blood, the determination of oxygen capacity was left for the last when often there was not enough blood for the technique to be carried out on more than one of the two or three samples. In seven of the later studies, however, both oxygen capacity and content were determined on each sample of blood. From the data the degree of saturation of hemoglobin was calculated. The results appear in Table III.

In all the experiments the oxygen capacity of the blood is greater during and after exercise. The capacity is increased in blood drawn as early as the 2nd minute of exercise (H.E.H., October 27, 1922). The results are in accord with previous observations. As long ago as 1888, Cohnstein and Zuntz (9) noted an increase in the number of red blood corpuscles during exercise. Geppert and Zuntz (1) found an increase in capacity after tetanization of their dogs. Hawk (10), in 1904, found the corpuscles most numerous at the beginning of exercise (after $\frac{1}{2}$ to 3 minutes of swimming) with a gradual decrease as the exertion continued.

TABLE II.
Changes in Oxygen Content as a Result of Exercise.

Subject.	Date.	Amount and duration of exercise.	O ₂ content before exercise.	Time of blood taking during or after exercise.	O ₂ content during or after exercise.	Difference before and after exercise.
	<i>1921</i>					
J.E.	Nov. 2	3,700 kg. in 3½ min.	20.3	3 min. after.	22.5	2.2
N.P.L.	" 15	3,595 " " 3½ "	23.1	3 " "	21.0	0.9
A.B.	Dec. 7	2,675 " " 2½ "	20.4	3 " "	21.4	1.0
P.R.	" 10	3,695 " " 3½ "	20.2	3 " "	21.0	0.8
	<i>1922</i>					
M.K.	Mar. 24	489 " " 2½ "	18.9	3 " "	20.0	1.1
H.E.H.	" 29	3,055 " " 3½ "	20.1	3 " "	20.9	0.8
M.L.	Apr. 7	3,285 " " 3½ "	21.3	3 " "	22.4	1.1
H.B.R.	" 12	3,500 " (about) in 3½ min.	20.6	3 " "	21.5	0.9
H.E.H.	June 15	4,350 " in 4 min. 10 sec.	18.9	3 " "	20.1	1.2
D.P.B.	Aug. 8	3,770 " " 3½ "	19.7	3 " "	21.4	1.7
D.P.B.	" 15	3,820 " " 3½ "	19.9	3 " "	22.7	2.8
	<i>1921</i>					
D.C.M.	Nov. 23	3,575 " " 3½ "		3 " "	23.4	
	" 30	3,605 " " 3½ "	21.1	15 " "	22.2	
J. McL.				3 " "	22.4	1.3
	<i>1922</i>			8 " "	22.1	1.0
H.E.H.	Apr. 5	3,954 " " 3½ "	19.6	1 " "	21.0	1.4
	" 21	4,104 " " 3½ "		3 " "	21.1	1.5
				15 " "	21.8	
				30 " "	20.9	
					19.9	

H.E.H.	Aug. 10	3,400 kg. in 3 $\frac{3}{4}$ min.	18.0	During last min. 3 min. after.	19.7	1.7
H.E.H.	" 25	3,545 " " 3 " 20 sec.	17.6	During last min. 3 min. after.	19.7	1.7
H.E.H.	Apr. 14	4,098 " " 4 "	19.4	During last min.	18.4	0.8
D.P.B.	June 21	7,380 " " 7 $\frac{1}{2}$ "	20.2	" " "	18.0	0.4
D.P.B.	Oct. 17	6,162 " " 5 " 22 sec.	20.6	5 min. after.	21.4	2.0
M.F.	" 19	4,074 " " 4 " 34 "	20.8	7 " "	23.8	3.6
H.E.H.	" 27	3,408 " " 3 $\frac{1}{2}$ "	20.0	During last min. 5 min. after. During 2nd min. 12 min. after.	22.7	2.5
					22.7	2.1
					22.0	1.2
					22.0	1.2
					21.2	1.2
					20.9	0.9

TABLE III.
Oxygen Content, Oxygen Capacity, and Saturation of Hemoglobin Before, During, and After Short Periods of Vigorous Exercise.

Subject.	Date.	Oxygen content.			Difference in oxygen content.			Oxygen capacity.			Difference in oxygen capacity.			Saturation of hemoglobin.			Difference in saturation of hemoglobin.		
		Before.	During.	After.	During.	After.		Before.	During.	After.	During.	After.		Before.	During.	After.	During.	After.	
		<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>		<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>	<i>vol.</i> <i>per cent</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
D.P.B.	Aug. 8	19.7		21.4	1.7	20.5		21.9			1.4	96.1		97.7			1.6		
H.E.H.	" 10	18.0	19.7	19.7	1.7	19.1	20.0	20.2	0.9		1.1	94.2	98.5	97.5		4.3	3.3		
D.P.B.	" 15	19.9		22.7	2.8	21.3		23.8			2.5	93.5		95.0			1.5		
H.E.H.	" 25	17.6	18.4	18.0	0.8	0.1	19.3	18.9			1.6	93.2	95.3	95.2			2.6		
D.P.B.	Oct. 17	20.6		22.7	2.1	22.1		23.7						95.8					
M.F.	" 19	20.8	22.0	22.0	1.2	1.2	22.9	23.0					96.1	95.7					
H.E.H.	" 17	20.0	21.2	20.9	1.2	0.9	21.1	21.6			0.5	94.8		96.8			2.0		

As a result of exercise both oxygen content and oxygen capacity rise. It is significant that the increase in content is always greater than that of capacity though the difference between the rise in content and capacity is in no individual experiment beyond the limits of experimental error. In all the observations, however, a higher saturation of hemoglobin is indicated. The increase is possible because of the fact that hemoglobin is not completely saturated at rest. Meakins and Davies (11) observed an average saturation of 95.3 per cent and Harrop (4) of 95.5. In the five cases in which we calculated oxygen saturation before exercise, the average was 94.4 per cent.

Fatiguing Exercise.—In addition to the experiments on short periods of work included in Tables II and III, two later observations were made on exertion of more fatiguing character and of longer duration. D.P.B. (April 18, 1923) did stationary running and vigorous arm exercise for 7 minutes to the point of subjective fatigue. The oxygen content of his blood rose from 21.3 volumes per cent before exercise to 21.9 volumes per cent 5 minutes after the exertion. In the other experiment, H.E.H. (May 17, 1923) continued work of similar character for 14 minutes. Oxygen content was 20.4 volumes per cent before exercise and 20.5 volumes per cent 1 minute after the cessation of work. The capacity rose from 20.8 to 22.1 volumes per cent. Hence the hemoglobin saturation fell from 98.1 to 92.8 per cent.

DISCUSSION.

Factors Influencing Oxygen Content.—The results of our experiments with short periods of moderate exertion are in agreement with those of Geppert and Zuntz (1) and of Hastings (2). In all cases, following such exercise, there is an increased arterial oxygen content and apparently a greater saturation of hemoglobin in the arterial blood. Conceivably, these results might be considered an evidence of oxygen secretion, but even the protagonists of this theory do not claim that secretion exists in untrained persons doing moderate work at sea-level. An attempt at explanation on any other basis must take into account many of the numerous factors which determine the absorption and utilization of oxygen in the body. For the sake of convenience in discussion, these have been presented diagrammatically in Fig. 1.

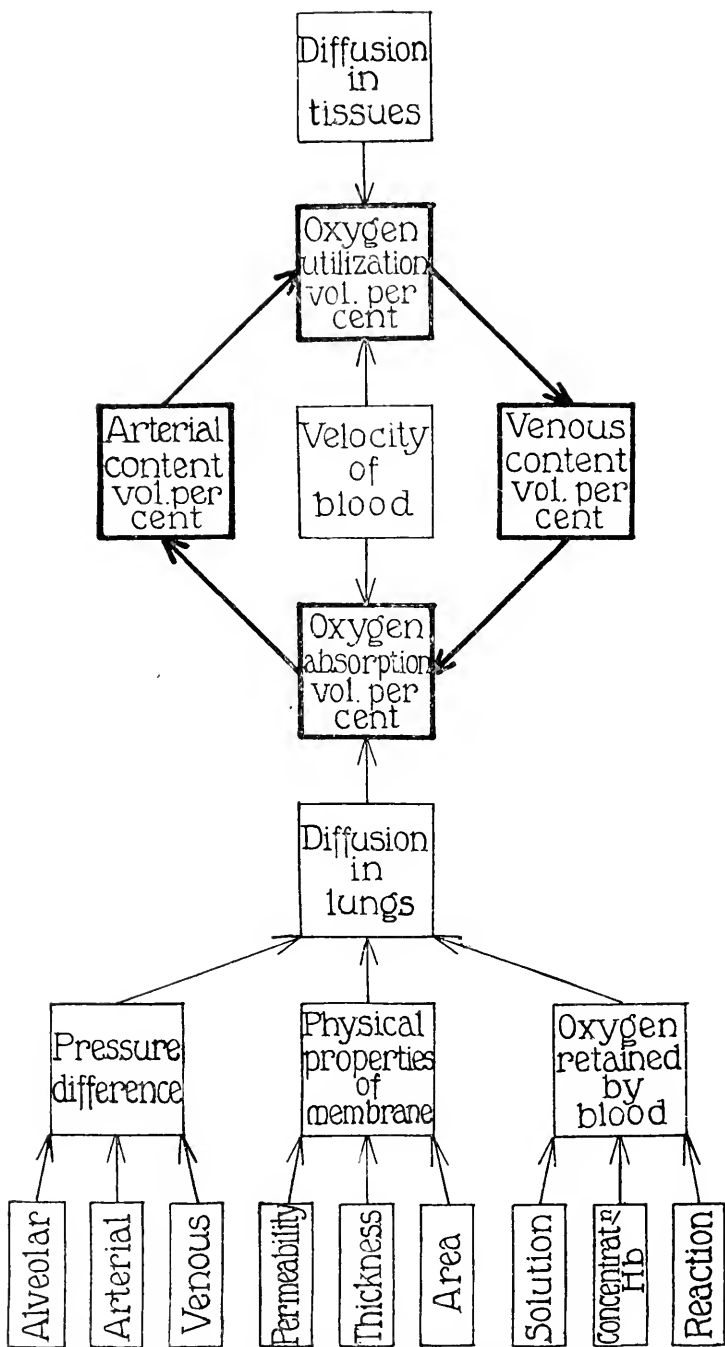


FIG. 1. Factors influencing oxygen content of arterial blood.

The heavily lined squares of the diagram show simply the gain and loss of oxygen during the circulation of the blood. Oxygen is lost in the tissues and regained in the lungs. Thus the oxygen content of both arterial and venous blood depends primarily upon the relative amounts of oxygen utilized in the tissues and of oxygen absorbed in the lungs. The *utilization* of oxygen (the amount diffusing out of each unit of blood in the tissues) is determined by the total oxygen diffusion through the systemic capillaries and by the velocity of the blood stream. Meakins and Davies (11) have demonstrated that with an increased blood flow, less oxygen is given off from each unit of blood. For the present discussion it is not necessary to consider the many influences which affect the total oxygen diffusion in the tissues. The *absorption* of oxygen (the amount added to each unit of blood in the lungs) depends upon the total volume of oxygen diffusing through the pulmonary membrane per minute and the rate of blood flow. The greater the velocity, the larger will be the volume of blood to share the incoming oxygen and the less will be the portion of each unit of blood.

Total diffusion of oxygen in the lungs per minute depends upon several factors which are represented in the diagram: (1) the pressure forcing oxygen through the membrane, (2) the volume of oxygen the alveolar membrane allows to pass per minute, and (3) the amount of oxygen each unit of blood is able to retain. The *effective pressure* is the difference between the alveolar oxygen tension and the tension of the blood in the lung capillaries which will depend upon the *venous* and *arterial* tensions. The *physical properties* (permeability, thickness, and area) of the alveolar membrane determine the amount of oxygen which may pass per unit time with a given pressure difference. Whatever the permeability of an individual's alveolar membrane may be, the thinner it is and the larger its area, the greater will be the amount of oxygen which can diffuse in a given time. The *oxygen retained* by each unit of blood depends in small part upon the solubility of oxygen in plasma, but much more upon the combination of oxygen with hemoglobin. The amount of oxygen with which hemoglobin may combine, is chiefly determined by its concentration, but is influenced by the reaction of the blood, a diminished alkalinity causing a decrease in the oxygen-combining power of hemoglobin at any physiological tension.

Short Periods of Exercise at Sea-Level.—The increase in arterial content found in our experiments during and after exercise might be due to a decrease in the oxygen utilization in the tissues or to an increase in the oxygen absorbed in the lungs. The experiments of Zuntz and Hagemann (12), Krogh and Lindhard (13), Lindhard (14), and Douglas and Haldane (15) demonstrate that, in spite of the more rapid blood flow the utilization of oxygen is increased and the mixed venous blood returns to the lungs with a diminished load of oxygen. The greater arterial content must be attributed to a greater absorption of oxygen by the blood in the lungs. Furthermore, the oxygen added must be greater in amount than that lost in the tissues. The more rapid circulation rate during exercise tends to diminish the volume of oxygen which is received by each unit of blood. Hence the only way to account for a higher oxygen content is by a great increase in the total diffusion through the lung membrane per minute. What factors tend to cause such an increase in exercise at sea-level?

1. Each unit of blood may be able to retain more oxygen. Our experiments and those of others have demonstrated a considerable increase in the amount of hemoglobin in the blood during and after exercise. This alone would tend to increase total diffusion. Its effect, however, is partly neutralized by the diminished alkalinity of the blood which occurs as the result of vigorous exertion. The final effect of these two opposing factors on the oxygen-combining power at any given tension must be found by experiment. Even if the ability of the blood to retain oxygen were not diminished by an acidosis, the increase in capacity due to the concentration of hemoglobin could not entirely explain the higher content, for the increase in content is greater than that in capacity. The only other way that arterial content may rise is by an increase in the oxygen tension of the blood. Either of the two factors next to be considered may raise arterial oxygen tension.

2. Greater diffusion may be caused by a larger difference between alveolar and blood tension of the lung capillaries. Due to increased utilization of oxygen in the tissues, there is always a fall in the tension of venous blood during vigorous exercise. Douglas and Haldane (15) concluded that the greater diffusion

which occurred in their exercise experiments was largely the result of the fall in venous tension. The increase in diffusion caused by falling venous tension can raise arterial tension at best only to the level of that in the alveoli. On the other hand, a rise in alveolar tension during work might increase the arterial above its resting value. Owing to the technical difficulties of obtaining alveolar samples during exercise the values for alveolar oxygen tension are not known. Following exertion, however, the marked fall in CO_2 tension observed by Douglas and Haldane (16) and others indicates that the oxygen tension of the alveolar air is considerably increased. With the cessation of exercise, this may be an important factor in raising arterial tension and content.

3. The hyperpnea of exercise may also raise arterial tension by increasing the alveolar area. Bohr (17) found that the hyperpnea of exercise produces a larger mean alveolar volume and suggests that it is a mechanism for securing more oxygen. Krogh and Lindhard (18) did not always observe this increase in the first minutes of work. If there is a larger volume which is accompanied by a stretching of the alveolar membrane, as was suggested by M. Krogh (19), then the membrane becomes thinner and more permeable to oxygen. A larger volume may be due to the opening of more alveoli for according to Haldane, Meakins, and Priestley (20) deep breathing causes the disappearance of the atelectasis which, they say, exists in some parts of the lungs during the more shallow breathing at rest.

The preceding discussion may be summarized as follows: With vigorous but not exhausting exercise at sea-level certain factors tend to diminish oxygen content of arterial blood, some tend to increase it, while the influence of others is unknown. The greater utilization of oxygen in the tissues causes the venous blood to return to the lungs with less oxygen. This must be more than made up by the oxygen diffusion in the lungs. The more rapid blood flow in the pulmonary circuit during exercise tends to diminish the volume of oxygen taken up by each unit of blood, hence, any rise in content must be due to an increased total diffusion of oxygen. Of the various factors which influence total diffusion the diminished alkalinity is known to limit it, while the greater concentration of hemoglobin and the diminished

oxygen tension of venous blood tend to raise it. The rise in content may also be due in part to an increase in alveolar area and alveolar oxygen tension as well as to a decrease in the thickness of the membrane. Our results for short periods of moderate exercise at sea-level indicate that the factors which augment oxygen diffusion outweigh the effect of the increase in circulation rate.

Exercise at Altitudes.—Barcroft and his coworkers (3) found a lower oxygen content during exercise, a result exactly opposed to ours. Since his work, however, was performed under low oxygen pressure, it seemed possible that the conditions may have modified the influence of the various factors which have been discussed. At altitudes or in a low pressure chamber, the total diffusion through the lung membrane may not be increased as much by muscular exertion because many of the mechanisms for securing a greater supply of oxygen during exercise at sea-level are being employed to obtain more oxygen during rest at altitudes.

1. There is a concentration of hemoglobin at altitudes under resting conditions. The observations of Schneider and Havens (21, 22) indicate that there may be little further increase in hemoglobin as the result of short periods of exercise soon after reaching a great height. Barcroft's (3) oxygen capacity was not determined before exercise, but it is likely that the increase which resulted from exertion was less than ours. The failure to increase hemoglobin concentration does not, however, explain the lower oxygen content of the arterial blood. This may be accounted for by the factors discussed below.

2. The low oxygen pressure at altitudes produces an hyperpnea during rest. If this increases the mean alveolar volume at rest, the possible rise due to work is diminished. Alveolar surface may not increase to the same degree as at sea-level. In a similar manner any increase in alveolar tension and decrease in thickness of the membrane are limited.

3. Another compensation for acquiring oxygen during rest at altitudes is a decrease in venous oxygen tension. Doi (23) has shown that the saturation of hemoglobin in arterial and mixed venous blood decreased with a diminishing oxygen content of the inspired air. The resting tension of Barcroft's mixed

venous blood must have been lower than at sea-level. Under these circumstances, venous tension might fall to a minimum even with a small amount of work. With more vigorous exertion, the additional oxygen may be no more than sufficient for the greater needs of the tissues, with the result that the venous blood remains at a minimum. After this stage, the pressure difference in the lungs and the total diffusion of oxygen is no longer increased by falling venous tension. The same amount of oxygen passing the membrane is diffused, because of the more rapid blood flow, into a greater volume of blood. There is a resulting decrease in arterial tension and content.

The fall in arterial tension tends to improve diffusion. However, the increase in the rate of diffusion from this cause is slight because the pressure difference between alveolar and arterial tensions is small.

These deductions are based on the data of Barcroft and his coadjutors (3) and an analysis of the factors shown in Fig. 1. M. Krogh (19), in 1915, gave a similar explanation for the results of Douglas, Haldane, Henderson, and Schneider (24) at Pike's Peak even though at that time direct determinations of the oxygen content of the arterial blood were not available.

Though arterial tension falls, the other factors which improve diffusion at sea-level, the increased concentration of hemoglobin, diminished venous tension, greater alveolar surface, and tension and diminution of the thickness of alveolar membrane, are less active at altitudes. The increase in total diffusion is not as great, while the blood flow may be as rapid, as it is for an equivalent amount of work at sea-level. It appears that arterial content is chiefly determined by the opposing effects of total oxygen diffusion and the rate of blood flow. When the diffusion increases more than the circulation rate, as in the experiments at sea-level, the oxygen content of arterial blood rises. When the blood flow increases to a greater degree than diffusion, as in Barcroft's observation, a fall in arterial content results.

Severe Exertion at Sea-Level.—The fall in arterial content at altitudes indicates that the circulation rate continues to increase after diffusion has reached a maximum. Does the same phenomenon obtain under any circumstances in the normal man at sea-level?

There is evidence that with continued severe exertion there may be a reduction in oxygen tension and saturation of hemoglobin, and even of content. In three experiments on Dog II who was a poor runner, Hastings (2) could never obtain a higher content, and in one determination the content fell 0.5 volume per cent. Harrop (4) found a decrease of 3.1 volumes per cent in arterial content after 15 minutes of severe exertion.

D.P.B. (April 18, 1923) exercised strenuously for 7 minutes. The oxygen content in blood drawn about 5 minutes after exertion rose 0.6 volume per cent. This is in contrast to a previous result of D.P.B. (June 21, 1922). Here, too, the blood was drawn 5 minutes after $7\frac{1}{2}$ minutes of exercise, but the work was of a less strenuous character. The content increased 2.5 volumes per cent. It is interesting to note that for the less exhausting work the same subject's average increase in content was 2.2 volumes per cent with a minimum of 1.7 volumes per cent.

In the experiment on H.E.H. (May 17, 1923) when severe exertion was continued for 14 minutes, there was no change in the oxygen content of arterial blood taken during the 2nd minute after exercise. The oxygen capacity increased as usual so that the saturation of hemoglobin decreased. The latter fact indicates that the oxygen tension diminished.

The complete evidence for moderate and severe exertion at sea-level seems to warrant the following hypothesis: The value of arterial content goes through two stages—a rise and a fall. At first diffusion improves more rapidly than the circulation rate, and arterial content rises. As diffusion approaches a maximum it increases more slowly, so that a continued increase in circulation gradually lowers arterial content, and with severe and prolonged exertion the content may be reduced even below its resting value. In this case, exertion may be limited because of an insufficient diffusion of oxygen through the membrane rather than because of an inadequate circulation rate.

The discrepancy in the findings in the literature occurs because some observations were obtained in the first stage and others in the second. The difference between the results at altitude and sea-level is due to the fact that diffusion cannot achieve as great a maximum at altitudes. Hence, the second stage is attained with the comparatively slow circulation produced by a smaller amount of work.

Anoxemia and Hyperpnea.—Harrop's (4) observation upon the effect of exhaustive exercise lends support to the view that work may produce an arterial anoxemia which may stimulate the respiratory center. Our findings with severe exertion and Barcroft's results at altitudes are susceptible to the same interpretation. In all these experiments a fall in arterial oxygen tension is indicated. On the other hand, the findings of Geppert and Zuntz (1) and Hastings (2) as well as our own observations with less exhausting exercise show that during vigorous exertion at sea-level diffusion may be increased to such an extent that both O₂ content and tension rise in the arterial blood. In these observations no part of the hyperpnea can be explained by an arterial anoxemia.

SUMMARY AND CONCLUSIONS.

1. In twenty experiments on normal men at sea-level, doing short periods of vigorous work on a bicycle ergometer, oxygen content rose above the resting value during and after exercise.

2. In seven experiments, both oxygen capacity and oxygen content were determined. There was a rise in both after exertion, but the content was increased more than the capacity, indicating a greater degree of oxygen saturation in the arterial blood.

3. Since both oxygen content and saturation of hemoglobin are higher after vigorous exercise, no part of the hyperpnea from exertion in these experiments can be attributed to an anoxemia in the arterial blood.

4. In two experiments, the effect of more fatiguing exertion was observed. In one there was a slight rise in content in blood drawn during the 5th minute after exercise. In the other, the content was the same in the 2nd minute after exercise as before. The capacity rose as usual so that the saturation of hemoglobin, and presumably the oxygen tension, decreased.

5. In exhausting exercise it is possible that an anoxemia in the arterial blood may play a part in the causation of hyperpnea.

6. The conflicting results in the literature may be explained by the following hypothesis: During exercise, arterial content is chiefly determined by the opposing effects of oxygen diffusion in the lungs and the velocity of the blood stream. In the normal man, arterial content may go through two stages if the

exertion is severe. Early in exercise diffusion improves more rapidly than the circulation rate, and arterial content rises. Later, diffusion increases slowly, so that the continued rise in circulation rate reduces arterial content. It is possible that the severity of the exercise which a normal individual will tolerate may be determined by the amount of oxygen which can diffuse through the lung membrane rather than by the capabilities of the circulation.

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BIOCHEMICAL STUDIES ON ALLANTOIN.

I. THE INFLUENCE OF AMINO-ACIDS ON THE EXCRETION OF ALLANTOIN BY THE RABBIT.*

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While a considerable amount of work has been done on the influence of the ingestion of non-purine foods upon the endogenous purine metabolism, most of the experiments have been carried out upon human subjects, probably because short and accurate methods have been available for the determination of uric acid, the end-product of purine metabolism in man. For allantoin, on the other hand, the methods of determination are long and tedious and probably not as accurate as the methods for uric acid. A review of the literature shows that comparatively few studies have been made on the relation of diet to the endogenous purine metabolism of those animals which excrete allantoin as the final purine catabolite.

The greater part of the work which has been done on the factors which influence allantoin excretion has been concerned chiefly with an effort to find definite precursors of the purine bodies. Since histidine and allantoin both contain the glyoxaline ring Abderhalden and his pupils (1) thought that a synthesis of allantoin might follow the ingestion of histidine. The administration of 10 gm. of histidine hydrochloride *per os* to a dog failed to increase appreciably the elimination of allantoin. Ackroyd and Hopkins (2), on the other hand, showed that the removal of histidine and arginine from the diet of growing rats caused a marked loss in body weight, accompanied by a decreased elimination of allantoin. The addition of histidine and arginine to the diet caused a renewal of growth and an increase in allantoin excretion. There was some decrease in allantoin elimination when one of the acids was removed, but not so much as

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when both acids were absent from the diet. A diet deficient in its tryptophane content, while it caused nutritional failure, did not appreciably influence allantoin excretion. From these experiments, the authors concluded that arginine and histidine were synthesized to purines. Harding and Young (3) noted a marked increase in the excretion of allantoin in puppies when the protein of the diet, previously supplied by meat powder, was changed to protein prepared from placenta. Since the total nitrogen excretion on both diets was about the same, they considered the factor of stimulation by amino-acids to be negligible. A chemical analysis of the two proteins showed that they differed little in preformed purine content, but that the placental material was much richer in arginine and histidine. Hence these authors concluded that their work was in accord with that of Ackroyd and Hopkins in demonstrating that arginine and histidine form the raw material for the synthesis of the purine ring.

It is now agreed that in man, the elimination of endogenous uric acid, the end-product of purine metabolism, is not a constant quantity but varies with the kind and amount of food ingested, even though the food be largely free from purine. Proteins ingested in large amounts may cause a considerable increase in the endogenous uric acid elimination, while fats and carbohydrates exert a less marked influence. A careful study of the influence of protein and its hydrolytic products, the amino-acids, upon uric acid excretion has been made by one of us (4). It was demonstrated that amino-acids influence uric acid excretion in a manner similar to proteins, which would seem to rule out the work of the digestive glands as an important factor. It was suggested that the effect is due to a general stimulation of cellular metabolism by amino-acids or their products of catabolism. Rose (5) has recently discussed this theory and has submitted evidence in its support as have also Gibson and Doisy (6).

In view of these studies on man and since there is little in the literature relative to the excretion of allantoin after high protein diets or amino-acid ingestion, it has seemed of interest to study this further by the administration of proteins and amino-acids to rabbits and the determination of their effect on allantoin elimination. It was thought that results might be obtained which would be of value in connection with the comparative chemistry of purine metabolism.

The experiments have shown that *in the organism of the rabbit, the ingestion of considerable amounts of amino-acids (glycine, alanine) resulted in a marked drop in the daily elimination of*

allantoin, a result which is entirely different from that obtained in man, in whom the ingestion of amino-acids increased the excretion of uric acid, which has a metabolic significance in man similar to that of allantoin in the rabbit.

EXPERIMENTAL.

The first series of experiments reported in this paper was carried out in the spring of 1922 and a second series in the spring of 1923. In the earlier studies allantoin was determined by Harding and Young's (3) modification of the method of Plimmer and Skelton (7). The results which were obtained in the first experiments were so unexpected, that it was considered advisable to repeat the work with another and possibly more accurate method for the estimation of allantoin. Accordingly, the determinations of allantoin in the later studies were made by the Handovsky (8) modification of the method of Wicchowski (9).

Each sample of urine in the first series (Tables I to IV) was analyzed daily for total nitrogen, urea and ammonia nitrogen, "Folin" nitrogen, creatinine, and, in some experiments, uric acid. Total nitrogen was determined by the Kjeldahl-Gunning method; creatinine, which was used as a check on the completeness of the collection of the urine, by the micro method of Folin; and uric acid, by the method of Morris and Macleod (10). 10 cc. of urine were used in the latter determination because of the small amount of uric acid in rabbit urine. The "Folin" nitrogen was determined by the Harding and Young (3) procedure. The term, "Folin" nitrogen, as used in this study refers to the nitrogen determined by the magnesium chloride-hydrochloric acid hydrolysis in the Folin (11) procedure for the estimation of urea. It has been shown that this treatment not only hydrolyzes urea but allantoin as well. The ammonia formed in the reaction together with the preformed ammonia is distilled from alkaline solution into an excess of standard acid and the amount of nitrogen determined. The urea and ammonia were determined by the urease method of Van Slyke and Cullen. The difference between the "Folin" nitrogen and the urease nitrogen gives a measure of the allantoin nitrogen.

The method as outlined by Harding and Young (3) was followed closely with two exceptions. An ordinary thistle tube was attached to the upper end of the condenser, but the bent glass tube at the top of the thistle tube was omitted since our experiments did not show it to be of any value. The thistle tube was not put in place until the contents of the flask were evaporated to the point of foaming.

In order to test out the accuracy of the method for the determination of allantoin, a number of analyses were made on mix-

tures of pure allantoin and other urinary constituents. It was found that the allantoin in solutions of allantoin and urea could be quantitatively recovered, but that a small amount of creatinine

TABLE I.

Rabbit A, male, white. Daily diet: 100 cc. of milk, 25 gm. of oats, and 15 gm. of hay.

Date.	Weight.	Total N.	Urea N.	Allantoin.	Creatinine.	Remarks.
1922	kg.	mg.	mg.	mg.	mg.	
Mar. 31	2.50	912	805	70	136	
Apr. 1	2.50	897	791	66	153	
" 2	2.50	844	751	53	150	
" 3	2.52	849	737	79	141	
" 4	2.52	1,274	1,087	30	143	5 gm. glycocoll.
" 5	2.52	1,596	1,346	0	127	5 " "
" 6	2.53	942	845	49	145	
" 7	2.52	926	805	83	142	
" 8	2.53	1,018	902	71	141	
" 9	2.52	867	761	74	138	
" 10	2.52	1,695	1,521	117	179	10 gm. gelatin.
" 11	2.52	1,818	1,703	65	150	10 " "
" 12	2.55	1,848	1,732	29	128	10 " "
" 13	2.56	1,082	956	86	129	
" 14	2.56	1,131	1,010	67	137	
" 15	2.56	978	894	47	137	
" 16	2.56	1,008	896	52	140	
" 28	2.56	954	830	71	141	
" 29	2.57	936	816	68	144	
" 30	2.57	969	855	69	144	
May 1	2.60	909	797	72	132	
" 2	2.60	1,359	1,249	7	137	5 gm. glutamic acid as the sodium salt.
" 3	2.61	1,360	1,243	26	138	5 gm. glutamic acid as the sodium salt.
" 4	2.60	827	710	65	137	
" 5	2.60	933	816	55	140	
" 6	2.60	879	755	71	137	

nitrogen was also broken down in the hydrolytic treatment, when the latter constituent was added to a mixture of the first two. Allantoin added to urine was also quantitatively recovered. During the course of the experiments, it occurred to us that

certain results which were obtained might be due to an inaccuracy of the method, when the urea nitrogen was doubled and the allantoin nitrogen presumably remained the same. To test out this point, a sample of rabbit urine was analyzed for total nitrogen, "Folin" nitrogen, and urease nitrogen. Then urea was added to approximately double the nitrogen content of the urine and the

TABLE II.

Rabbit B, male, blue-grey. Daily diet: 100 cc. of milk, 25 gm. of oats, and 15 gm. of hay.

Date.	Weight.	Total N.	Urea N.	Uric acid.	Allantoin.	Creatinine.	Remarks.
1922	kg.	mg.	mg.	mg.	mg.	mg.	
Apr. 27	2.10	870	741		65	105	
" 28	2.12	800	686		51	98	
" 29	2.15	891	764		71	115	
" 30	2.13	765	644		58	98	
May 1	2.13	1,482	1,332		71	95	10 gm. gelatin.
" 2	2.20	1,863	1,730		31	120	10 " "
" 3	2.20	1,770	1,642		39	107	10 " "
" 4	2.17	852	724		90	102	
" 5	2.15	899	758		55	112	
" 6	2.15	855	738		63	97	
" 7	2.12	841	724		69	101	
" 15	2.23	887	769	4.3	70	107	
" 16	2.20	899	783	4.6	77	107	
" 17	2.20	936	830	4.7	50	120	
" 18	2.20	1,206	1,110	4.4	13	101	5 gm. glutamic acid as the sodium salt.
" 19	2.23	1,289	1,172	5.8	14	100	5 gm. glutamic acid as the sodium salt.
" 20	2.23	946	843	5.6	26	105	
" 21	2.23	833	741	4.9	25	100	
" 22	2.20	870	766	4.7	56	103	
" 23	2.25	864	788	6.7	70	110	
" 24	2.25	854	796	7.4	64	113	

analysis repeated. The amount of allantoin found was the same in both cases. From these experiments it was concluded that the method of Harding and Young was sufficiently accurate for metabolism studies although the figures for allantoin might be somewhat high due to the partial hydrolysis of creatinine or other urinary constituents. This error, however, would vary

little from day to day, as the excretion of creatinine is usually constant. In addition to the quantitative tests a qualitative test for sugar was made by the Benedict method. Plimmer and Skelton (7) have pointed out that a modification of their method must be used if sugar is present in the urine as the sugar causes the formation of humin nitrogen and gives low results. No

TABLE III.

Rabbit C, male, grey. Daily diet: 100 cc. of milk, 25 gm. of oats, and 15 gm. of hay.

Date.	Weight.	Total N.	Urea N.	Uric acid.	Allantoin.	Creatinine.	Remarks.
<i>Day.</i>	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
May 19	2.21	891	788	6.8	83	110	
" 20	2.23	865	741	7.0	77	108	
" 21	2.25	884	780	6.8	69	98	
" 22	2.23	920	810	7.4	58	110	
" 23	2.22	1,589	1,498	9.2	26	112	10 gm. gelatin.
" 24	2.22	1,662	1,578	10.0	18	105	10 " "
" 25	2.25	1,832	1,726	11.0	45	110	10 " "
" 26	2.26	1,906	1,809	13.2	25	107	10 " "
" 27	2.27	920	821	8.6	82	98	
" 28	2.27	884	774	8.6	88	113	
" 30	2.25	913	838	8.8	82	101	
" 31	2.25	870	771	7.9	50	95	
June 1	2.26	1,626	1,576	7.6	25	82	3 gm. urea.
" 2	2.25	1,855	1,781	7.8	11	100	3 " "
" 3	2.27	1,515	1,471	8.6	18	98	3 " "
" 4*	2.25	511	491	8.4	6	74	
" 5	Animal died.						

* Sample probably not complete for this date.

reduction of Benedict's solution greater than that given by normal urines was given by any of the urines analyzed in this study.

In the latter part of the work (Tables V to IX), as already stated, the Handovsky (8) modification of the method of Wiechowski (9) was used for the determination of allantoin. The procedure finally adopted for the determination of allantoin was as follows:

The 24 hour sample of urine was diluted¹ to a volume of 250 cc. 100 cc. portions of the diluted urine were treated with 5 cc. of glacial acetic acid and 50 cc. of 1 per cent sulfuric acid and made up to a volume of 250 cc. This entire volume was treated with 10 gm. of phosphotungstic acid and allowed to stand for several hours. The resulting filtrate gave no precipitate with phosphotungstic acid except on long standing. The bulk of the filtrate from the phosphotungstic acid precipitate was then treated with lead oxide (PbO) until a creamy white precipitate settled out leaving a clear supernatant liquid. This solution after filtration gave no precipitate with basic lead acetate and reacted slightly alkaline. As a rule 175 cc.

TABLE IV.

Rabbit D, male, black. Daily diet: 100 cc. of milk, 25 gm. of oats, and 15 gm. of hay.

Date.	Weight.	Total N.	Urea N.	Uric acid.	Allantoin.	Creatinine.	Remarks.
<i>1922</i>	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
June 24	2.30	522	433	5.5	90	72	
" 25	2.30	865	754	9.0	92	105	
" 26	2.00	815	697	9.8	83	99	
" 27	2.00	825	699	7.7	67	99	
" 28	2.10	1,819	1,689	18.6	17	111	2.5 gm. urea.
" 29	2.00	1,771	1,554	8.5	8	96	2.5 " "
" 30	1.98	813	702	8.1	59	104	
July 1	1.98	636	537	9.4	76	89	
" 2	1.97	801	677	10.3	95	98	
" 3	2.00	1,350	1,123	10.5	2.9	94	5.0 gm. glycocoll.
" 4	2.10	1,464	1,054	10.8	55.0	87	5.0 " "
" 5	1.98	884	813	6.6	21.0	91	
" 6	2.00	686	585	8.5	87.0	92	
" 7	1.98	727	622	9.0	66.0	88	

of this filtrate were recovered. The chlorides were next removed by the addition of 5 cc. of glacial acetic acid and 20 cc. of a solution of silver acetate. After filtration the heavy metals were precipitated with hydrogen sulfide and the sulfides were filtered off. The excess of hydrogen sulfide was removed by aeration and the solution was neutralized with calcium carbonate (Baker's c.p.). The solution was again filtered to remove the excess of calcium carbonate and the filtrate was then aerated with air free from carbon dioxide, to remove the excess of carbon dioxide. This solution gave no precipitate with either phosphotungstic acid, basic lead acetate, or silver acetate. As a rule 100 cc. of this filtrate were treated

¹ Urea determinations were made on the urines of the experimental days. In no case was the concentration of urea in the diluted urines greater than that recommended for the determination of allantoin by Wiechowski.

with 100 cc. of the reagent used for the precipitation of allantoin (0.5 per cent mercuric acetate in 20 per cent sodium acetate). After 30 minutes the solution was filtered and 50 cc. portions of the filtrate were titrated with 0.1 N potassium thiocyanate to determine the excess of mercuric acetate in the filtrate. None of the filtrations was made by suction and consequently smaller aliquots of the original volume were recovered than would have otherwise been obtained. 100 cc. of the filtrate, before the addition of the mercury reagent, represents seven-twentieths of the original volume of urine taken for analysis (100 cc.).

TABLE V.

Rabbit 3, male, black. Daily diet: 125 cc. of milk and 30 gm. of oats.

Date.	Weight.	Allantoin.	Creatinine.	Remarks.
1923	kg.	mg.	mg.	
Mar. 30	2.70	187	87	
" 31	2.70	150	53	
Apr. 1	2.70	138	76	
" 2	2.70	184	83	
" 3	2.70	184	78	10 gm. gelatin.
" 4	2.70	187	89	10 " "
" 5	2.70	187	75	
" 6	2.70	143	71	
" 7	2.75	136	64	
" 8	2.70	87	77	5 gm. glycecoll.
" 9	2.75	16	74	5 " "
" 10	2.70	174	72	
" 11	2.75	143	72	
" 12	2.70	142	75	
" 13	2.70	163	78	
" 14	2.70	109	75	5 gm. urea.
" 15	2.70	109	71	5 " "
" 16	2.70	118	67	
" 17	2.70	Sample lost.		
" 18	2.70	157	75	
" 19	2.70	143	69	
" 20	2.65	149	73	
" 21	2.65	120	75	5 gm. alanine.
" 22	2.65	108	78	5 " "
" 23	2.65	127	75	
" 24	2.65	150	75	
" 25	2.65	161	73	

Prior to the use of the method for the metabolism studies, numerous experiments were conducted to test out the accuracy of the method. It was shown that when pure allantoin solutions

were treated with an excess of the precipitating reagent, the allantoin could be quantitatively recovered. However, when allantoin was added to human urine and the analysis carried out as described previously, the recoveries averaged from 85 to 95 per cent. It was considered possible that the final filtrate from

TABLE VI.

Rabbit 4, male, black. Daily diet: 125 cc. of milk and 30 gm. of oats.

Date.	Weight.	Allantoin.	Creatinine.	Remarks.
<i>1923</i>	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	
Mar. 30	2.80	213	94	
" 31	2.80	162	81	
Apr. 1	2.80	146	77	
" 2	2.80	221	94	
" 3	2.80	182	86	10 gm. gelatin.
" 4	2.80	209	98	10 " "
" 5	2.80	209	81	
" 6	2.80	174	83	
" 7	2.80	170	80	
" 8	2.80	166	77	5 gm. urea.
" 9	2.80*	157	84	5 " "
" 10	2.80	212	83	
" 11	2.80	174	76	
" 12	2.80	202	92	
" 13	2.75	163	81	
" 14	2.75	48	81	5 gm. glycocoll.
" 15	2.75	13	73	5 " "
" 16	2.75	149	76	
" 17		Sample lost.		
" 18	2.75	172	82	
" 19	2.75	159	78	
" 20	2.65	174	81	
" 21	2.65	179	86	2 gm. glycocoll.
" 22	2.65	139	84	2 " "
" 23	2.65	172	80	
" 24	2.65	194	85	
" 25	2.65	194	83	

* Slight albuminuria.

some of the experimental urines, which did not give the usual heavy white precipitate (complex mercury allantoin compound) upon the addition of the mercuric acetate reagent might still contain allantoin that failed to precipitate because of improper conditions for precipitation. However, it was found that if

TABLE VII.

Rabbit 5, male, black. Daily diet: 125 cc. of milk and 30 gm. of oats with a small carrot and a small amount of cabbage leaves.

Date.	Weight.	Allantoin.	Creatinine.	Remarks.
<i>1923</i>	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	
May 2	3.10	181	83	
" 3	3.05	211	100	
" 4	3.00	245	100	
" 5	3.05	219	100	
" 6	3.05	104	98	5 gm. glycocoll.
" 7	3.05	114	100	5 " "
" 8	3.05	191	86	
" 9	3.05	199	76	
" 10	3.05	178	87	
" 11	3.00	147	98	6 gm. alanine.
" 12	3.00	70	90	6 " "
" 13	3.00	184	86	
" 14	3.00	179	92	
" 15	3.00	168	83	

TABLE VIII.

Rabbit 6, male, white and black. Daily diet: 125 cc. of milk, 30 gm. of oats, 100 gm. of carrots, and 50 gm. of cabbage.

Date.	Weight.	Total N.	Allantoin.	Creatinine.	Remarks.
<i>1923</i>	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
May 21	2.45		112	71	
" 22	2.45		153	76	
" 23	2.45		131	58	
" 24	2.10		156	66	
" 25	2.45	2,403	142	62	10 gm. gelatin.
" 26	2.10	2,733	150	60	10 " "
" 27	2.40		112	56	
" 28	2.35		174	66	
" 29	2.35		168	66	
" 30	2.35		187	66	
" 31	2.35		37	66	5 gm. glycocoll in one dose.
June 1	Animal found dead in cage.				

the mercury-allantoin compound was filtered off, the filtrate always gave an immediate precipitate, if a few drops of a 1 per cent solution of allantoin were added, indicating that conditions for the precipitation of allantoin were satisfactory. Moreover, it was shown, that in those urines in which the amount of the final mercury-allantoin precipitate was small, allantoin added to the filtrate prior to the addition of the mercuric acetate reagent could be quantitatively recovered.

TABLE IX.

Rabbit 7, male, white. Daily diet: 40 gm. of oats, 150 gm. of carrots, and 100 gm. of cabbage.

Date.	Weight.	Total N.	Allantoin.	Creatinine.	Remarks.
1923	kg.	mg.	mg.	mg.	
May 21	2.75		100	67	
" 22	2.70		138	73	
" 23	2.70		131	71	
" 24	2.75		137	75	
" 25	2.80	1,683	82	70	10 gm. gelatin.
" 26	2.80	2,823	105	73	15 " "
" 27	2.80		87	72	
" 28	2.80		62	69	
" 29	2.80		162	70	
" 30	2.80		156	64	
" 31	2.80	1,510	137	72	5 gm. glycocoll.
June 1	2.80	1,563	62	64	5 " "
" 2	2.80		118	56	
" 3	2.80	880	112	72	
" 4	2.80	738	131	80	
" 5	2.80	885	129	75	
" 6	2.80*	753	105	68	4 gm. cystine.
" 7	2.70†				2 " "
" 8	Animal died.				

* Albuminuria.

† Marked albuminuria and casts.

The animals used in all the experiments were male rabbits weighing between 2 and 3 kilos. The daily diet varied with individual rabbits and may be found in the tables.

In all the experiments reported, the substances studied were administered orally through a gastric sound. The gelatin was dissolved in water to form a thick syrup. In some experiments

this was further diluted with water while in other experiments it was diluted with a portion of the daily allowance of milk. Glycocoll, alanine, and urea were likewise either dissolved in a small quantity of water or in the milk. Glutamic acid was administered as a solution of its sodium salt and cystine in neutral suspension. At the same hour each day the bladder was emptied by gentle pressure and this urine added to that collected from the cage. In most cases the analysis of the urine was begun at once and if this was not possible the urines were preserved with chloroform or toluene and kept in an ice box until the completion of the analysis. The results are found in Tables I to IX.

DISCUSSION.

It will be seen from an inspection of the tables that the rabbits of the first series of experiments (Tables I to IV) excreted normally a smaller amount of allantoin per day than the rabbits of the last set of experiments (Tables V to IX). With the exception of Rabbit A (Table I) the rabbits used in the earlier work were somewhat smaller than those of the later experiments. Moreover, the daily diet of Rabbits A, B, C, and D (Tables I to IV) included 15 gm. of dried hay, which was entirely lacking in the diet of Rabbits 3 to 7 (Tables V to IX). As stated previously, the analyses of allantoin in the earlier work were made by the method of Harding and Young (3), while the method of Handovsky (8) was used in the later work. It seems hardly probable that the difference in the methods of analysis can explain the variation in the normal level of allantoin excretion in the two groups of rabbits. In a recent paper by Stransky (12), rabbits of nearly uniform weight and on uniform diets showed marked individual differences with respect to the elimination of allantoin. These differences can hardly be explained by the methods of analysis since the method of Handovsky (8) was used throughout.

A marked decrease in allantoin elimination followed the administration of amino-acids and in some cases, of gelatin and of urea. 5 gm. of glycocoll daily were given on 2 successive days to Rabbits A (Table I), D (Table IV), 3 (Table V), 4 (Table VI), 5 (Table VII), and 7 (Table IX). A 2.5 gm. portion was given during the 1st hour of the experimental day and 5 hours later a second 2.5 gm. dose was administered. In every case, it will be observed

that there was a marked decrease in the elimination of allantoin. It is to be noted in the case of Rabbits A and D that this condition of lowered elimination persisted 1 day after the last feeding of glycocoll. Rabbit 4 (Table VI) was given 2 gm. of glycocoll daily in a single dose to determine whether decreased elimination of allantoin would follow the ingestion of smaller amounts of amino-acids. It was also thought possible that a smaller dose might cause an increased elimination of allantoin similar to the increased uric acid elimination in man following amino-acid ingestion. The results indicated that on the 1st day of glycocoll feeding there was no apparent effect, but that there was a 20 per cent decrease in the elimination of allantoin on the 2nd feeding day. 5 gm. of glycocoll were given to Rabbit 6 (Table VIII) at one feeding. Only one 24 hour sample of urine was collected as the animal died on the following day. Again, however, the excretion of allantoin was markedly lower than on the days of regular diet. Rabbit A (Table I) and Rabbit B (Table II) received 5 gm. daily of glutamic acid as the sodium salt for 2 successive days. In both cases there was a marked drop in the allantoin elimination and in Rabbit B the condition of lowered excretion continued for 2 days after the last feeding of the glutamic acid. After the administration of a total of 10 gm. of alanine to Rabbit 3 (Table V) over a period of 2 days, there followed a decrease of 30 per cent in the elimination of allantoin. Rabbit 5 (Table VII) received daily feedings of 6 gm. of alanine for 2 successive days. The allantoin elimination was decreased 20 per cent on the 1st day and 60 per cent on the 2nd day of feeding. 4 gm. of cystine were fed to Rabbit 7 (Table IX) in one dose. The elimination of allantoin was somewhat decreased as compared with the normal. 2 gm. of cystine were fed on the following day, but the animal died before a complete 24 hour sample of urine could be obtained. The urine on both experimental days contained large amounts of protein and the sample obtained after the second feeding of cystine contained numerous casts. In all the experiments the urines were tested daily for the presence of protein and this was the only case where a distinctly positive test for its presence was obtained. In one other sample of urine (Rabbit 4, Table VI) there was a slight albuminuria following the administration of urea.

The results of the administration of gelatin on the elimination of allantoin were not as uniform as those following the feeding of amino-acids. Rabbit A (Table I) was given a total of 30 gm. of gelatin during a period of 3 days. The allantoin excretion on the 1st day was increased 50 per cent. On the 2nd day of gelatin feeding the excretion was normal, but on the 3rd day, the allantoin elimination was only 50 per cent of the normal. Rabbit B (Table II) also received 30 gm. of gelatin over a period of 3 days. The excretion of allantoin was apparently not affected by the first 10 gm. of gelatin but on the 2 following days there was a marked decrease in allantoin excretion. Rabbit C (Table III) was given 10 gm. of gelatin daily for 4 successive days and had a lowered elimination of allantoin throughout the entire 4 days of the experiment. The allantoin excretion of Rabbits 3 and 4 (Tables V and VI) was apparently not affected by the administration of 10 gm. of gelatin daily for 2 days. Rabbit 6 (Table VIII) showed a slightly decreased elimination on the 1st day of normal diet following the ingestion of 20 gm. of this protein. Rabbit 7 (Table IX) received a total of 25 gm. of gelatin in 2 days and there was a decreased elimination of allantoin on the days of feeding which persisted 2 days after the animal was on a normal diet. In summary, four out of seven rabbits showed a marked decrease in allantoin elimination following the ingestion of gelatin. One rabbit excreted less allantoin on the 1st day after the last feeding and in two rabbits the elimination of allantoin was not affected.

Since the feeding of protein and amino-acids in the amounts used in these experiments resulted in the elimination of large amounts of urea, it was thought that the excretion of allantoin might be affected by the administration of urea in much the same way as by the feeding of proteins and amino-acids. Rabbit C (Table III) received a total of 9 gm. of urea over a period of 3 days. For this 3 day period the total excretion of allantoin was less than the average excretion of allantoin for a single day, when the animal was on a normal diet. Rabbit D (Table IV) was given 2.5 gm. of urea per day for 2 days. The allantoin excretion was lowered considerably on the 2 experimental days, but returned to the normal amount on the 1st day of regular diet. Following the ingestion of 5 gm. of urea daily for 2 days by Rabbit 3 (Table V) there was a decrease in allantoin elimination of 30 per cent.

The allantoin elimination of Rabbit 4 (Table VI) was unchanged by the daily administration of 5 gm. of urea during a period of 2 days. Whether the decreased elimination of allantoin is closely associated with the abnormally large excretion of urea by the animal cannot be definitely decided from the present data. An examination of Tables I to IV will show that there are several instances, where the conditions of lowered allantoin elimination persisted after the urea nitrogen had returned to its normal level.

Since it was thought that the decreased allantoin excretion might be compensated for in part by an increased excretion of uric acid, determinations of the latter constituent were made in some experiments. In only one case (Rabbit C, Table III) following the administration of gelatin, did there appear to be a constant increase in uric acid above the normal. Since the increases in absolute amounts of uric acid were so small no conclusions could be drawn.

There are few experiments cited in the literature, where a decreased allantoin excretion is associated with a high protein diet.

An examination of the experiments in which Abderhalden and his co-workers (1) fed 10 gm. of histidine monochloride to a dog excreting normally 1.6 gm. of nitrogen per day showed that as the nitrogen increased to 2.5 gm., due to the nitrogen of the histidine, there was an increase in allantoin excretion from 98 to 114 mg. per day. However, on the following day, with a nitrogen excretion of 2.2 gm. and above the normal level, the dog excreted only 64 mg. of allantoin. On the following 3 days as the nitrogen output gradually returned to the former level of 1.6 gm. per day, the allantoin excretions on the corresponding days were 52, 28, and 10 mg., respectively. A second paper (13) from the same laboratory was concerned with the administration of histidine monochloride to a fasting dog. The first administration of 10 gm. of the histidine compound failed to raise the nitrogen on the day of feeding, but the allantoin was decreased by 75 per cent. However, the nitrogen output increased on the following 2 days with an elimination of allantoin 50 per cent above the normal. The second 10 gm. dose of histidine monochloride, given 5 days after the first administration, was followed by an increase in the total nitrogen elimination from 1.8 to 3.8 gm. and a decrease in allantoin elimination from 205 to 152 mg. On the following days, although the total nitrogen excretion was lowered to the normal level, the allantoin excretion remained approximately the same (150 mg. daily). On the other hand, Benedict (14) reported one experiment in which the increase in the total nitrogen of the diet of a Dalmatian hound from 5.9 to 24.1 gm. caused an increase in allantoin elimination of from 60 to 100 mg. The conditions, however, of his

experiments and the experiments reported in this study are not analogous since the Dalmatian hound normally excretes a larger percentage of its purine catabolites as uric acid and a smaller amount as allantoin.

The results obtained in this study are so completely at variance with the studies on endogenous purine metabolism in man following the ingestion of protein and amino-acids in excessive amounts that their explanation is somewhat difficult.

It is to be noted that the amounts of amino-acids fed to rabbits in these experiments are much greater in proportion to the weight of the rabbits than the amounts of amino-acids which produced an increase in uric acid excretion in man. It is possible that, in excessive amounts, the amino-acids exert some toxic effect within the cell, which influences the course of purine metabolism. Starkenstein (15) has shown that the behavior of the corresponding purine catabolites, allantoin in the rabbit and uric acid in man, do not necessarily run parallel. He has demonstrated that the effects of atophan upon the excretion of allantoin in rabbits and upon that of uric acid in man are quite different, since atophan causes a decreased elimination in the former and an increased elimination in the latter case.

It is the purpose of the present paper to present the data which we have obtained. Further experiments are in progress which it is hoped will throw further light on the comparative endogenous purine metabolism.

SUMMARY.

1. The feeding of amino-acids (glycocoll, alanine, and glutamic acid) to rabbits was followed by a marked decrease in the daily elimination of allantoin.

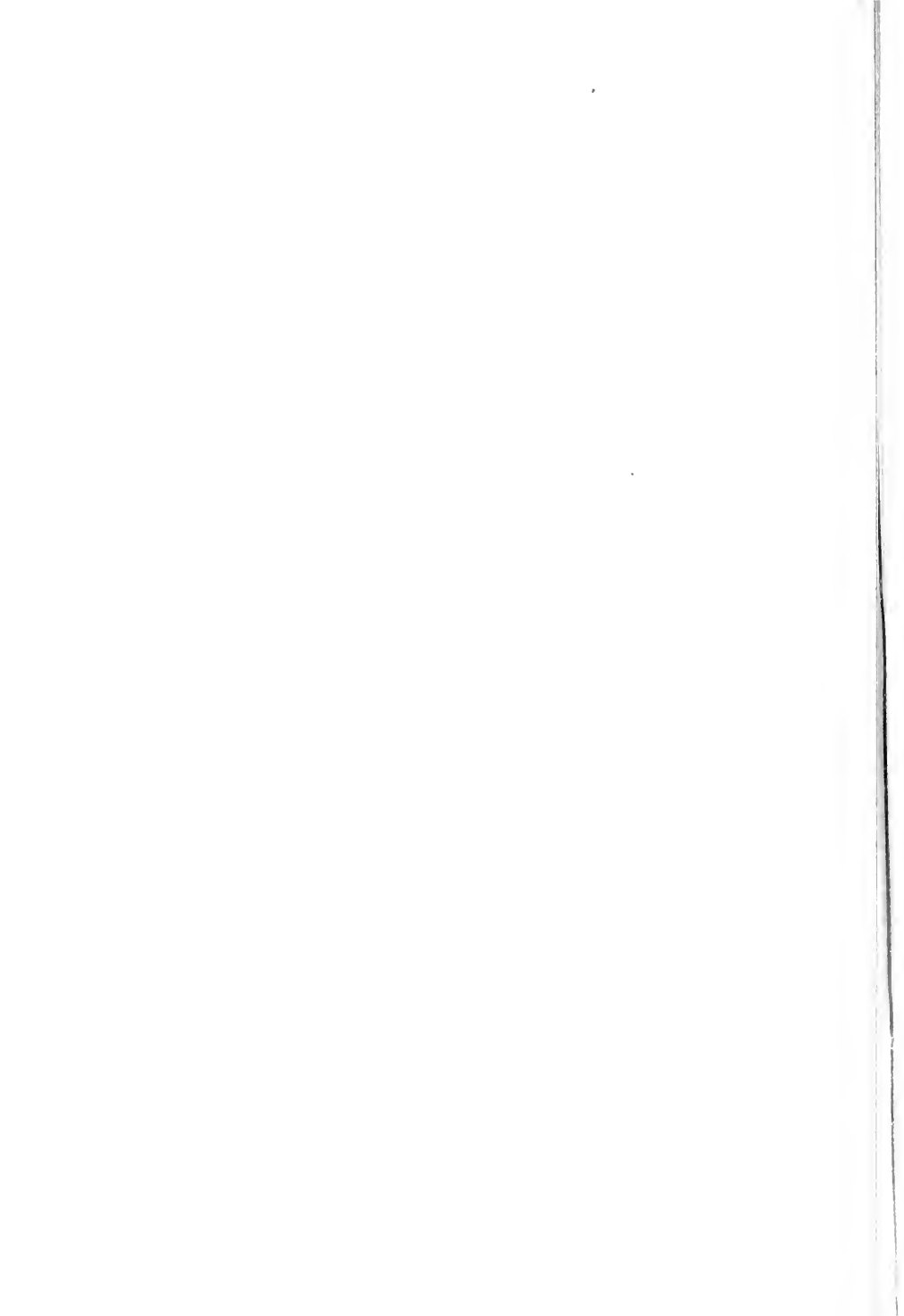
2. Similar results were obtained after enteral administration of large amounts of gelatin. The changes observed, however, were neither so constant nor so striking.

3. The effects of urea feeding on allantoin excretion were similar to those produced by the feeding of amino-acids.

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THE GROWTH OF YEAST ON A MEDIUM OF WHOLLY SYNTHETIC ORIGIN.

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Previous communications from this laboratory (1, 2) have maintained that continuous growth of yeast is possible on media composed of salts and cane-sugar. MacDonald and McCollum (3) and MacDonald (4) hold the same opinion.

The fact that one of the constituents of the media, cane-sugar, is of natural origin leaves open to criticism any definite conclusions in regard to the need of bios for the growth of yeast. No matter how much the sugar had been purified, if the yeast grew, it might be argued that the successful growth of the organism was due to unanalyzable traces of bios still retained by the sugar. Robertson and Davis (5) and Willaman and Olsen (6) stress this point.

Whether the successful use of cane-sugar was due to traces of bios could be answered by the use of some synthetic source of carbon and energy as a substitute for the cane-sugar. The material used in the experiments here described was first made by Loew (7) and was named "methose" by him. He states that the product is fermentable by yeast.

Methose was prepared in this laboratory as follows: The formalin was acidified with sulfuric acid and distilled, the acid preventing the formation of non-volatile products. The distillate was made up to contain the following per liter: 10 gm. of formaldehyde, 0.125 gm. of magnesium oxide, 0.50 gm. of magnesium sulfate, and 75 gm. of granulated lead. The material was heated on the water bath at 65-70° until the odor of formaldehyde had disappeared, this point being reached in a day or two. The lead was filtered off and the liquid evaporated *in vacuo* to small volume, made up to 80 per cent ethyl alcohol, and the lead

and magnesium salts were precipitated out with carbon dioxide. After filtration the liquid was evaporated on the water bath. The resulting material was a colorless or slightly yellow syrup which was used in the following experiments as a substitute for cane-sugar.

The yeast used had been growing in medium E (2) for $3\frac{1}{2}$ years and came originally from a cake of Fleischmann's yeast and is designated as *Saccharomyces cerevisiae* Raeb. F. Medium E contained per 100 cc.: 0.188 gm. of ammonium chloride, 0.100 gm. of dipotassium phosphate, 0.100 gm. of calcium chloride, 0.04 gm. of calcium carbonate, and 8 gm. of cane-sugar.

In the following experiments the yeast was grown in a medium containing 2 gm. of methose per 100 cc. and no cane-sugar, the rest of the medium being identical with medium E mentioned above. All data were obtained at 30°C. At each transferral enough yeast was added to make an initial count of one (when the count is one there are 250,000 cells per cc.). After 36 to 72 hours the count after each successive subculture was: 79, 39, 42, 42, 109, and 184. Since the original count in each case was one, the product of the above numbers will give the dilution of the original yeast as about 1:10.¹¹ The medium was diluted 1:25 each time, making the greatest possible "carry over" about 1:10.⁶ There can be no question that the yeast was growing only on the constituents of the medium and, therefore, that the success in the subculture of yeast on cane-sugar could not have been due to traces of impurities unless the same or similar impurities were synthesized along with the methose.

CONCLUSION.

Yeast has been subcultured on a medium wholly synthetic in origin.

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AN ELECTROCHEMICAL STUDY OF HEMOGLOBIN.

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In the course of a series of investigations on the physical chemistry of hemoglobin now being carried on in the Laboratory of Physical Chemistry of the Harvard Medical School, Dr. E. J. Cohn and Dr. R. M. Ferry discovered certain abnormalities in the behavior of a hydrogen electrode immersed in solutions of hemoglobin and oxyhemoglobin. On studying the matter further they found that any inert electrode when dipped into a hemoglobin solution gave a fairly definite potential which varied with the degree of oxygenation of the hemoglobin. It was at first thought that this potential might be the oxidation-reduction potential of the system hemoglobin-oxyhemoglobin, and in order to investigate this possibility the present work was undertaken by the author. It was soon found that the titration method of determining oxidation potentials, now being applied in this laboratory to a study of quinones (1, 2) and first proposed by Clark (3), was applicable to hemoglobin solutions; the system of oxidized and reduced compounds involved, however, was not oxyhemoglobin-hemoglobin, but hemoglobin-methemoglobin. The reactions which are the basis of the method are: hemoglobin + $\text{K}_3\text{Fe}(\text{CN})_6 \rightarrow$ methemoglobin; and 2 methemoglobin + $\text{Na}_2\text{S}_2\text{O}_4 \rightarrow$ 2 hemoglobin.

Procedure.

The experiments were performed in an apparatus identical with the one previously employed in this laboratory in our studies of anthraquinone derivatives (1). A glass cell holding 200 to 300 cc. of solution was fitted with a mechanical stirrer, gas inlet and outlet tubes, and three electrodes, and was connected to a saturated calomel electrode by an agar-potassium chloride bridge. The hemoglobin solution was mixed with a suitable buffer solution or

the requisite salts were dissolved in it so that the total salt concentration was about 0.2 M. This solution was placed in the cell which was swept out with nitrogen. Definite increments of reducing agent (sodium hydrosulfite) or oxidizing agent (potassium ferricyanide) were added from a burette, and the potentials of the electrodes were determined after each addition. In this way a titration curve was obtained such as is shown in Fig. 1. As might be expected, considering the complexity of the hemoglobin molecule, the experimental difficulties were much greater than those experienced with even such substances as the anthraquinones. In order to be sure that the potentials recorded were significant equilibrium potentials it was necessary to be certain that they were constant for at least 15 minutes, and that the potentials of at least two of the three different electrodes were identical. Several hours were required before equilibrium was reached in many cases. The three electrodes employed were platinized platinum, bright platinum, and gold. Certain electrodes gave erratic potentials and had to be discarded; soaking the electrodes in a solution of methemoglobin for several days seemed to improve them. Because of these difficulties the actual values of the potentials recorded in this paper must be considered as provisional and possibly to be in error by 30 or 40 millivolts.

A 0.1 N solution of potassium ferricyanide was used as the oxidizing agent; it must be kept away from the light. The sodium hydrosulfite solution which was employed was made up fresh every day and contained a small amount of sodium carbonate which greatly increased its stability. It was protected from oxidation in the burette by a layer of xylene. Each solution was standardized by a titration with 0.01 M indigo disulfonate which in turn was standardized against titanous chloride. (The titrations must be carried out in an atmosphere of carbon dioxide.) The hydrosulfite solution slowly changes in the burette and should be re-standardized at least every 6 hours.

The Hemoglobin-Methemoglobin Potential.

The hemoglobin used in this work was kindly furnished by the Laboratory of Physical Chemistry of the Harvard Medical School; it was prepared from horse blood according to the method of Ferry.¹

¹Ferry, R. M., *J. Biol. Chem.*, 1923, lvii, in press.

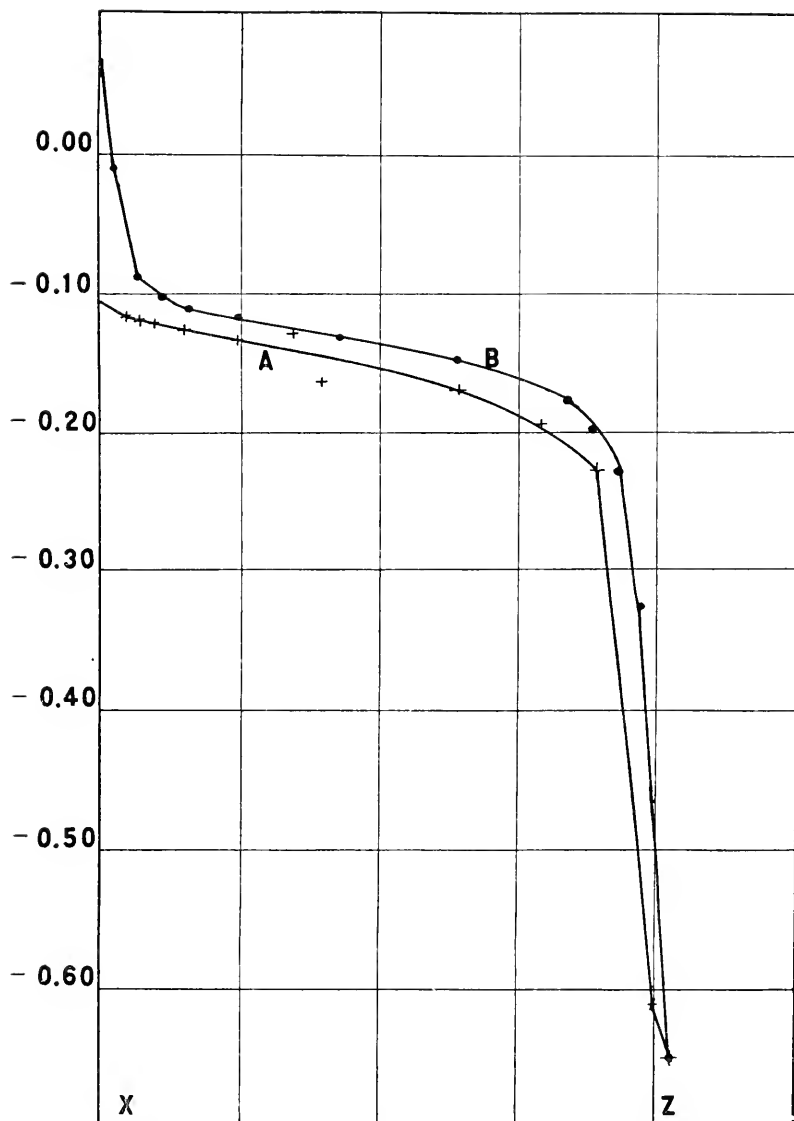


FIG. 1. Titration of hemoglobin and methemoglobin in pH 6.8. Potential against saturated calomel electrode plotted vertically; cc. of $\text{Na}_2\text{S}_2\text{O}_4$ plotted horizontally from x to z; cc. of $\text{K}_3\text{Fe}(\text{CN})_6$ from z to x. Curve A titration of methemoglobin with $\text{Na}_2\text{S}_2\text{O}_4$; Curve B hemoglobin with $\text{K}_3\text{Fe}(\text{CN})_6$.

A great variety of samples was used; some freshly prepared consisted almost wholly of oxyhemoglobin, while others which had stood several months in the ice chest contained a large proportion of methemoglobin. Except for the varying content of methemoglobin no difference was observed in the behavior of the various samples.

Titration can be carried out most conveniently by first adding small increments of ferricyanide to the hemoglobin solution (which usually contains varying amounts of oxy- and methemoglobin) until this reagent is present in excess as shown by the potential. Nitrogen is passed through the cell and then the titration with sodium hydrosulfite is commenced; the initial amount of ferricyanide necessary to convert all the material to methemoglobin is only of significance in regard to the relative amounts of oxy- and methemoglobin in the original sample. After the completion of the titration with sodium hydrosulfite, the hemoglobin can be titrated back to methemoglobin with ferricyanide. This titration back and forth can be repeated a number of times with the same material. Table I summarizes the results of a number of experiments. A nitrogen analysis of the hemoglobin sample employed in each experiment furnished the necessary information for determining the mols of hemoglobin present in the cell. (It was assumed that hemoglobin contained 17.3 per cent of nitrogen and that its molecular weight was 16,700.) From the amount of sodium hydrosulfite or ferricyanide required in a titration the number of mols of hemoglobin could be calculated. A good agreement was obtained between the amount of hemoglobin present according to the nitrogen analysis and according to the titrations calculated on the basis of 1 mol of Hb \approx 1 mol of $K_3Fe(CN)_6$ and 2 mols of Hb \approx 1 mol of $Na_2S_2O_4$. A comparison of the values in Column 2 and in Columns 5 and 9 of Table I, shows the agreement to be within our present experimental error (about 10 per cent). *The change from reduced hemoglobin to methemoglobin involves only one hydrogen equivalent of oxidizing or reducing agent per gram-molecule, calculated on a molecular weight of 16,700.*

Table II gives the actual potentials of the three electrodes in two of the titrations in Experiment 2. The curves in Fig. 1 are plotted from this table, the average potential being plotted against the amount of sodium hydrosulfite or ferricyanide added. It will be

observed that except for a few cases near the end-point the potentials of all three electrodes are quite close together. The potentials enclosed in brackets are obviously in error and were not included in the averages.

TABLE I.
Titration of Hemoglobin at $19 \pm 1^\circ$.

Experiment No.	Na ₂ S ₂ O ₄ titrations.					K ₃ Fe(CN) ₆ titrations.				
	Mols of Hb by analysis.	Molality of Na ₂ S ₂ O ₄ .	Required.	Mols of Hb by titration.	π_0	Molality of K ₃ Fe(CN) ₆ .	Required.	Mols of Hb by titration.	π_0	pH
			cc.				cc.			
1		0.0330	4.0 0.000268		+0.069					
	0.000281	0.0285	4.5 0.000258		+0.081	0.109	2.8 0.000306		+0.089	6.8
2		0.0285	44.0 0.00250		+0.124	0.109	20.0 0.00219		+0.114	
	0.00252	0.0328	39.0 0.00256		+0.104	0.109	21.5 0.00234		+0.114	8.5
3*	0.00165	0.0320	19.0 0.00122		+0.114	0.109	12.5 0.00136		+0.109	6.8
4	0.000675	0.0340	9.0 0.000612		-0.013	0.109	5.5 0.000600		-0.026	9.63
5	0.000447	0.0355	6.0 0.000427		-0.031	0.109	4.0 0.000440		-0.031	9.63
6	0.000224	0.0280	4.0 0.000222		+0.119					8.5
7*	0.000880	0.0375	10.0 0.000750		+0.024	0.109	7.5 0.000815		+0.021	9.63
8	0.000224	0.0360	3.5 0.000250		+0.029	0.109	2.0 0.000218		-0.081	11.3
9	0.000990	0.0224	21.0 0.000940		+0.029	0.100	9.5 0.000950		-0.014	11.3

A phosphate buffer solution was used for pH 6.8; a borate-boric acid solution for 8.5; a sodium hydroxide-borate solution for 9.63, and 0.2 M NH₄OH for pH 11.3. The total salt concentration in each case was about 0.2 M.

*Slight decomposition had occurred before the sample was used, judged by the odor.

The two curves (Fig. 1) showing the reduction with hydro-sulfite and the oxidation with ferricyanide are within 20 millivolts of each other. Such relatively consistent results are best obtained by using rather concentrated solutions of hemoglobin (about 10 per cent) and by having reliable electrodes.

Since, for the transformation of methemoglobin (MHb) to reduced hemoglobin (Hb), only one hydrogen equivalent is used per mol calculated on a molecular weight of 16,700, which in

TABLE II.
Titration of 0.00243 Mol of Hb in pH 8.5. Potentials Against Saturated Calomel Electrode.

Na ₂ S ₂ O ₄ 0.0325 molar.	Bright Pt.	Gold.	Platinized Pt.	Average potential.
cc.				
0.00	-0.105	-0.107	-0.106	-0.106
2.00	-0.116	-0.120	-0.114	-0.118
4.00	-0.120	-0.120	-0.120	-0.120
6.00	-0.125	-0.128	-0.126	-0.126
10.00	-0.128	-0.138	-0.131	-0.132
14.00	-0.121	-0.131	-0.132	-0.129
18.00	-0.136	-0.141	-0.143	-0.140
22.00	-0.154	-0.166	-0.164	-0.162
26.00	-0.170	-0.170	-0.172	-0.170
32.00	-0.190	-0.198	-0.192	-0.193
36.00	-0.234	-0.237	-0.211	-0.225
40.00	-0.628	-0.607	[-0.381]	-0.610
41.00	-0.672	-0.665	[-0.465]	-0.666

K₃Fe(CN)₆ 0.109 molar.

0.00	-0.672	-0.665	[-0.465]	-0.665
0.50	-0.532	-0.556	[-0.441]	-0.540
1.00	-0.307	-0.340	[-0.294]	-0.322
2.00	-0.207	-0.257	-0.222	-0.227
3.00	-0.189	-0.208	-0.185	-0.197
4.00	-0.163	-0.187	-0.164	-0.175
8.00	-0.141	-0.155	-0.142	-0.149
13.00	-0.132	-0.137	-0.129	-0.133
17.00	-0.118	-0.121	-0.117	-0.119
19.00	-0.110	-0.112	-0.110	-0.111
20.00	-0.099	-0.103	-0.103	-0.102
21.00	-0.084	-0.086	-0.096	-0.090
22.00	0.000	0.000	-0.033	-0.011
22.50	+0.079	+0.079	+0.045	+0.070

turn is based on the assumption of 1 iron atom per molecule, there is every reason to believe we are dealing with the change of a ferri compound to a ferro compound. In this event the potential is given by the equation:

$$(1) \quad \pi = \pi_o + \frac{RT}{F} \ln \frac{[\text{MHb}]}{[\text{Hb}]}$$

The value for the normal potential (π_o) is most conveniently obtained by interpolating the titration curve to the mid-point where $[\text{MHb}] = [\text{Hb}]$. The values thus obtained, referred to the normal hydrogen electrode, are given in Columns 6 and 10 of Table I. The present results are not sufficiently accurate to allow of a significant comparison of the slope of the titration curve with the theoretical slope calculated from the above equation.

The variations in π_o with change in the pH of the buffer solution require comment, although a more accurate determination of the values of π_o will be necessary before any very significant conclusions can be arrived at in this connection. If the activities of the oxidized and reduced compounds remain constant, the potential of the type represented by equation (1) should be independent of the hydrogen ion concentration. For the range pH 6.8 to 8.5 this seems to be the case for hemoglobin (within the present experimental error). There is a marked change in potential, however, between pH 8.5 and 9.6, and the potential is approximately constant again between pH 9.6 to 11.3. This change in potential at 8.5 to 9.6 may be caused by the formation of a salt by one of the acidic hydrogen atoms in the molecule which is present as the free acid in the range pH 6.8 to 8.5. If some such change takes place and the dissociation constants of this hydrogen in the reduced and oxidized form are different, a shift in the oxidation-reduction potential would take place. The following tabulation is a summary of the average values of π_o (referred to the hydrogen electrode) at different values of pH.

pH	π_o
	<i>volts</i>
6.8	+0.092 (± 0.022)
8.5	+0.115 (± 0.011)
9.63	-0.016 (± 0.040)
11.3	-0.025 (± 0.050)

Effect of Oxygen and Carbon Monoxide on the Potential.

It is thus evident that the potential of an inert electrode immersed in a hemoglobin solution depends on the relative amounts of reduced hemoglobin and methemoglobin present. The effect of passing air or carbon monoxide into such a mixture would be to diminish the amount of reduced hemoglobin present by forming oxy- or carboxyhemoglobin, and to raise the potential (the concentration of methemoglobin being unaffected by the presence of the gas). Removing the gas by passing in nitrogen should lower the potential again to its original value. These qualitative predictions have been verified by a number of experiments of which the following is typical.

The cell contained 175 cc. of a solution of hemoglobin, containing 2.6 gm. of hemoglobin per 100 cc. and mono- and disodium phosphates corresponding to pH 6.8. Sodium hydrosulfite was added until present in slight excess (as shown by the potential), the cell having previously been swept out with nitrogen. Air was then passed in for some time, the excess of hydrosulfite being rapidly oxidized and the potential rising to a value some 100 millivolts higher than the mid-point of the normal titration curve as shown by the following figures.

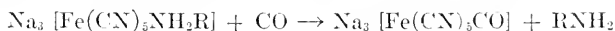
Nature of gas.	Equilibrium potential (referred to the hydrogen electrode).
	<i>volts</i>
Air.....	+0.239
Nitrogen.....	+0.149
Carbon monoxide.....	+0.300
Nitrogen.....	+0.139
Air.....	+0.250

If the reduced hemoglobin (either freshly prepared or after reduction with sodium hydrosulfite) is oxygenated by passing air through the cell and then titrated with sodium hydrosulfite 2 mols of hydrosulfite are used up per mol of hemoglobin. This corresponds to the 1 molecule of loosely combined oxygen in oxyhemoglobin. The potential during this titration is somewhat indefinite until the end-point is reached, but is usually at first some 100 or 200 millivolts above the normal titration curve. The dissolved oxygen in equilibrium with the oxyhemoglobin

apparently oxidizes the hydrosulfite as fast as it is added and the potential corresponds to the ratio of the small amount of methemoglobin to the relatively large amount of reduced hemoglobin.

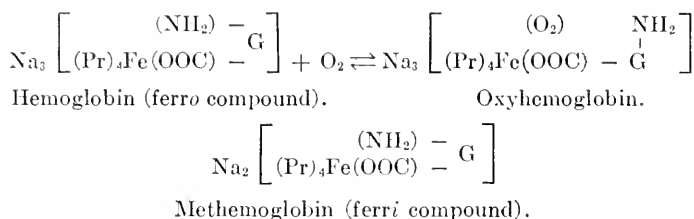
The potentials first noted by Dr. Cohn and Dr. Ferry in ordinary hemoglobin-oxyhemoglobin solutions can be explained in the light of the experiments just mentioned. The potential is due to the oxidation-reduction equilibrium involving methemoglobin and reduced hemoglobin, since there is probably a small amount of methemoglobin present in all hemoglobin solutions due to a slight decomposition of the oxyhemoglobin. By varying the oxygenation of such a mixture, the amount of free reduced hemoglobin is varied, and thus the potential is changed. That the potential is not due directly to the oxyhemoglobin is evident from the fact that carbon monoxide has the same effect as oxygen on the potential of a hemoglobin solution.

It seems quite evident that the oxygenation of hemoglobin is not an oxidation in the electronic sense, whereas the formation of methemoglobin is. When one looks for an analogous pair of simple ferri and ferro compounds stable in alkaline solution and reversibly oxidized and reduced, one is immediately struck with the similarity between hemoglobin and methemoglobin on the one hand, and sodium ferrocyanide and ferricyanide on the other. The work of Manchot (4) and Baudisch (5) makes this line of speculation still more tempting. Manchot found that ferro compounds of the type $\text{Na}_3[\text{Fe}(\text{CN})_5\text{NH}_2\text{R}]$ combine with carbon monoxide and nitric oxide in a fashion similar to hemoglobin. Baudisch believes the compound $\text{Na}_3[\text{Fe}(\text{CN})_5(\text{O}_2)]$ is formed under certain conditions, the oxygen being loosely held by one of the covalences of the iron in the same manner as the molecule of amine in Manchot's compound. As Manchot pointed out, his compounds differ from hemoglobin in their action with carbon monoxide only in that the absorption is irreversible.



If the amino group were part of a complex molecule joined at some other point to the central iron atom, the two products on the right of the above equation would be part of the same molecule and the reversible absorption of gas by a single molecule might be realized. The following formulas for hemoglobin, oxyhemo-

globin, and methemoglobin represent this idea. The four pyrrole rings (found in hematin) can be imagined as being the equivalent of 4 molecules of hydrocyanic acid,² the corresponding ions (represented by Pr) replacing 4 cyanide ions in Manchot's compounds. The fifth cyanide ion is replaced in these formulas by an acidic group of the globin molecule (G) while a free amino group of the same protein plays the rôle of the easily replaced amine.



While these formulas are, of course, speculative and incomplete it might be worth pointing out that they are in accord with several very different properties of hemoglobin. The relative ease with which the protein is split off from the hematin part of the molecule corresponds on the basis of these formulas to the breaking up of a complex iron salt and not to the hydrolysis of an amide linkage. The 3 hydrogen atoms of the complex ferro acid in hemoglobin (written above as the trisodium salt) by analogy with ferrocyanic acid ought to be moderately acidic and their dissociation constants would be presumably greatly affected by the nature of the six groups attached to the central atom. The strength of the tri-basic acids corresponding to hemoglobin and oxyhemoglobin should differ. Henderson (6) as a result of his study of the absorption of carbon dioxide by the blood believes that this must be the case. If the acidic hydrogen atoms involved in this change were part of ordinary acidic protein groups it is hard to see why the absorption of oxygen in some other portion of the molecule should materially affect their dissociation constants. The shift in the normal oxidation-reduction potential of hemoglobin referred to above, which seems to take place between pH 8.5 and 9.6, would be interpreted in terms of these formulas by the difference in the dissociation constants of one of the hydrogen atoms of

² There is considerable chemical similarity between pyrrole and hydrocyanic acid.

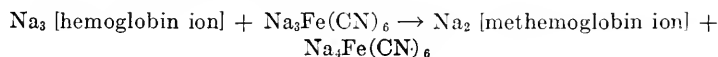
the ferro and ferri complex acids. It can be shown that if this is the case the potential of the hemoglobin-methemoglobin system would be given by equation (2) in which

$$(2) \quad \pi = \pi_o + \frac{RT}{F} \ln \frac{[\text{methemoglobin}]}{[\text{hemoglobin}]} + \frac{RT}{F} \ln \left(\frac{1 + \frac{[H^+]}{K_1}}{1 + \frac{[H^+]}{K_2}} \right)$$

K_1 and K_2 represent the dissociation constants of the weakest of the hydrogen atoms of the complex ferri acid and ferro acid, respectively. Such an equation as (2) would correspond to a shift in the oxidation-reduction potential over a rather small range of pH in which $[H^+]$, K_1 , and K_2 were of the same order of magnitude; for all other ranges the potential would be independent of the hydrogen ion concentration.

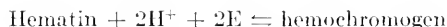
Ferricyanide Method of Determining Combined Oxygen.

The action of potassium ferricyanide on hemoglobin solutions which is the basis of Haldane's method for determining the combined oxygen, is readily interpreted now that the relation between hemoglobin and methemoglobin has been established. Reduced hemoglobin, oxyhemoglobin, and free oxygen are in equilibrium in the solution; the ferricyanide which is added oxidizes the reduced hemoglobin to methemoglobin, removing one of the components involved in the equilibrium. Oxyhemoglobin, therefore, dissociates into free oxygen and reduced hemoglobin as fast as the reduced hemoglobin is changed to methemoglobin, and the process continues until all the hemoglobin is converted to methemoglobin and the entire oxygen is liberated. Potassium ferricyanide is a peculiarly advantageous reagent for this oxidation as it is stable in alkaline solutions, has a high enough oxidation potential, and yet is not a sufficiently powerful oxidizing agent to attack the protein. The reaction between it and hemoglobin may be represented as follows:



Experiments with Hematin.

Hematin dissolved in solutions of pH 8.5 or greater alkalinity is reduced by sodium hydrosulfite to hemochromogen which in turn is oxidized to hematin by air or ferricyanide. The reduction with hydrosulfite may be followed by the change in potential in exactly the same manner as hemoglobin. The normal potential of this reversible reduction, however, is much lower than that of hemoglobin, being about $-0.153 (\pm 0.030)$ in pH 8.5, $-0.256 (\pm 0.020)$ in pH 9.6, and $-0.315 (\pm 0.010)$ in pH 11.3. The experimental difficulties in this electrometric titration seem to be fully as great as those encountered with hemoglobin and the results may be in error by 30 or 40 millivolts. Between 70 and 80 per cent of 1 mol of sodium hydrosulfite per mol of hematin was used up in each titration. This indicates that the reaction involves *two* hydrogen equivalents though the results cannot be considered as conclusive, particularly as it was not possible to obtain satisfactory titrations of hemochromogen with ferricyanide. The progressive change in the normal potential with change in pH further indicates that 2 hydrogen atoms are involved in the reduction which would be represented thus:



The potential of such a change (which is the usual quinone reduction) is given by the equation:

$$\pi = \pi_0 + \frac{RT}{2F} \log \frac{[\text{hematin}]}{[\text{hemochromogen}]} + \frac{RT}{F} \log [\text{H}^+]$$

The term $\frac{RT}{F} \log [\text{H}^+]$ in this equation corresponds roughly to the change in potential observed with variations in pH values.

Thus, the relation between hematin and hemochromogen seems to be similar to that between quinone and hydroquinone, the unsaturated system involving the pyrrole rings probably being the point of reduction. If this is the case the reduction of hematin bears no relationship to the reduction of methemoglobin nor of oxyhemoglobin, but finds its parallel in the reduction of the iron-

free hematoporphyrin to the leuco compound, which we have found can be brought about by vanadous chloride and is reversed by ferric chloride. This view of the structure of hemochromogen is contrary to Küster's ideas (7) according to which hemoglobin, oxyhemoglobin, and hemochromogen are ferro compounds, while methemoglobin and hematin are ferri compounds. Further investigations along this line are planned, although it would appear that this problem is but distantly related to the oxidation and oxygenation of hemoglobin.

I am greatly indebted to Dr. Cohn and Dr. Ferry, not only for enlisting my services in the study of the oxidation of hemoglobin but also for much advice and assistance in carrying out the present work.

SUMMARY.

1. The oxidation of hemoglobin to methemoglobin by potassium ferrieyanide and the reduction of methemoglobin to hemoglobin by sodium hydrosulfite can be followed electrometrically. The change involves one hydrogen equivalent and has a definite oxidation-reduction potential.

2. The potential of a mixture of hemoglobin and methemoglobin is raised by passing in oxygen or carbon monoxide, and lowered again by removing these gases completely. The potential of an inert electrode immersed in a hemoglobin solution varies with the partial pressure of the oxygen because the ratio of free hemoglobin to methemoglobin is varied by the degree of oxygenation. The change of hemoglobin to oxyhemoglobin is one involving oxygenation and not oxidation.

3. By representing hemoglobin as the sodium salt of a complex ferro acid, the relationships indicated by the present work can be adequately expressed and the analogy between hemoglobin and Manchot's amino-ferrocyanides emphasized.

4. Preliminary experiments with hematin seem to indicate that the reduction of this substance to hemochromogen involves the addition of 2 hydrogen atoms. If this is so, the relationship between this pair of compounds has no bearing on the problem of the oxidation or oxygenation of hemoglobin.

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STUDIES ON YEAST.

VII. THE DIETARY PROPERTIES OF YEAST.

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(Received for publication, May 21, 1923.)

Data presented in a previous communication (1) from this laboratory showed that yeast is a comparatively rich source of vitamin B. The statement was also made that animals receiving as low as 2.5 per cent of air-dried *Saccharomyces cerevisiae* grew at the normal rate, and that reproduction was possible on this amount of yeast in the diet as the sole source of vitamin B, although it was perhaps not normal. Kennedy and Palmer (2) have reported experiments which lead them to believe that yeast is not an unusually rich source of vitamin B. Apparently from their experiments the efficiency of yeast depends upon the manner in which the yeast is fed. They state, furthermore, that their animals were kept under favorable conditions for reproduction, but only in a very few instances were young produced, and these were never reared.

Data are presented in this paper which demonstrate that yeast is a rich source of vitamin B, not only for growth but for reproduction as well. This is of importance because yeast is used to a considerable extent as the sole source of vitamin B in experimental rations, and it is also being widely employed in experiments having for their object the isolation of this unknown dietary factor. Evans and Bishop (3) make the statement that animals fed their synthetic diets containing yeast as the sole source of vitamin B are for the most part sterile in the first generation and wholly so in the second generation. This apparent sterility could be overcome by the incorporation in the diet of small amounts of green leaves such as lettuce. They call this unknown dietary factor necessary for reproduction vitamin X.

Data are also presented on the nutritive value of yeast proteins.

EXPERIMENTAL.

Vigorous rats from 50 to 60 gm. in weight were used. The animals received an adequate growing ration supplemented with whole milk before they were placed on the experimental diets. The rations employed in all the experiments on the vitamin B content of yeast consisted of casein 18 per cent, salt mixture 18.5 (4) 3.7 per cent, filtered butter fat 5 per cent, yeast in various amounts from 1 per cent up to and including 8 per cent, and the remainder of the ration to 100 per cent was composed of dextrin. The dextrin was prepared by moistening starch with 1 per cent citric acid solution and autoclaving the mixture at 20 pounds pressure for 3 hours. The casein was purified by washing for several weeks with water acidified with acetic acid. No casein was employed which had not been tested to insure the absence of all vitamin B.

The yeast employed in these experiments contained no filler, and it is known as *Saccharomyces cerevisiae* (Fleischmann's Race F). In order to save space, the records of only a few of the many animals used will be given in the form of charts.

Value of Various Levels of Yeast for Growth and Reproduction.

Animals receiving 1 per cent of air-dried *Saccharomyces cerevisiae* as the sole source of vitamin B as a general rule give a growth curve which is below normal. None of the females has produced young on this amount of yeast, and only one of the twelve rats employed in this experiment has grown at the normal rate for a period of 14 weeks. A few of the animals have grown normally for a period of 2 to 3 months, but after that time the curve of growth has flattened out.

When 1.5 per cent of air-dried yeast is added to the diet, the curves of growth are better than when 1 per cent is used. Several of the rats have grown at about the normal rate over a period of 4 months or more. On the other hand, a few of the animals did not grow normally for such a long period of time. Two females produced young, but the young were undersized and died shortly after birth.

When 2 per cent and higher levels of air-dried yeast are incorporated in the diet, normal growth and reproduction result. In

order to save space only two charts recording the performance of animals on two different levels of yeast intake are given. Chart 1 shows the rapid growth of first generation animals on a diet containing 2.5 per cent of air-dried yeast as the sole source of vitamin B. The same types of growth curves and reproduction records were obtained on 2 per cent of air-dried yeast and upon levels of yeast greater than 2.5 per cent. The young were normal in weight when born, and the size of the litters was practically the same as on a good breeding ration. The reproduction records of

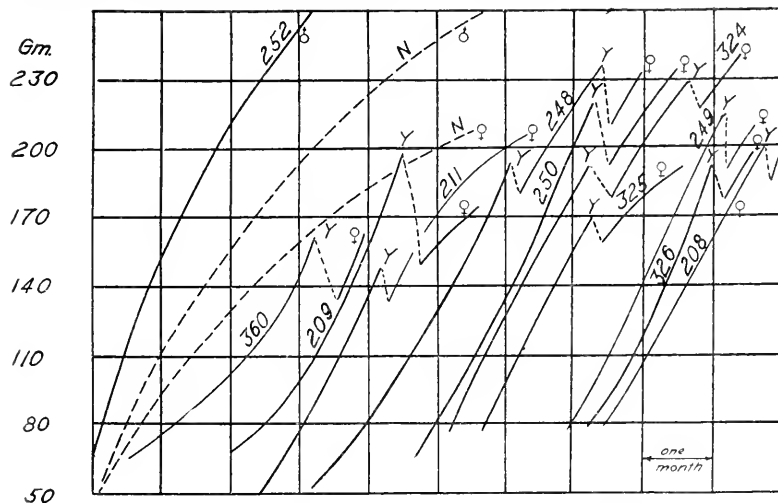


CHART 1. Curves of growth and reproduction records of animals whose diet contained 2.5 per cent of air-dried yeast as the sole source of vitamin B. There is no question of the capacity of these animals to reproduce. Only 6 of the 59 young produced on this ration have been reared.

all the animals are shown in Table I. The data presented in this table show that of the large number of young born on the various levels of yeast intake, only comparatively few were reared. The majority of the young died during the suckling period. The sixth column in Table I headed "Total No. of young that died" includes only the young which were cared for by the mothers in a normal manner, but notwithstanding this care died in an emaciated condition. The figures in this column do not include the young devoured by the mothers nor the young which were purposely

destroyed because the mothers either died or failed to care for the young. The number of young produced by all the animals totaled 308 and of this number 53 were reared to maturity. The table also shows that on 2.5 per cent of yeast fifteen young were reared, but only six of these young were brought to maturity on this amount of yeast; the remaining nine received tomato juice equivalent to 1.5 cc. per day in addition to the yeast. It was believed that some unknown constituent was missing from the diet which is necessary for normal milk production or normal milk composition

TABLE I.

Per- centage of yeast in ration.	No. of males on ration.	No. of females on ration.	No. of litters pro- duced.	Total No. of young born.	Total No. of young that died.	Total No. of young reared.	Young con- sumed by mothers.	Young that died for lack of care by mothers.	Young that died because mothers died.
1	4	7	0	0	0	0	0	0	0
1.5	3	7	2	12	6	0	0	6	0
2	1	4	4	28	12	0	16	0	0
2.5	10	14	11	59	35	15	0	5	4
3	5	4	5	40	33	0	0	0	7
3.5	6	10	6	42	14	14	14	0	0
4	1	3	1	7	7	0	0	0	0
5	9	14	12	58	12	24	10	12	0
8	1	4	4	16	12	4	0	0	0
4*	1	3	1	6	0	0	0	6	0
5*	3	7	5	24	23	1	0	0	0
6*	1	6	2	10	6	4	0	0	0
12*	1	3	1	6	6	0	0	0	0

* Signifies undried yeast containing 32 per cent of air-dried solids.

and so directly concerned in the successful nutrition of the young, and for that reason tomato juice was added. This small amount of tomato juice has been partially successful in preventing the high mortality of the young, but the same amount of orange juice does not improve the well being of the suckling.

Chart 2 shows the growth curves of second generation animals receiving 5 per cent of air-dried yeast as the only source of vitamin B in the diet. It will be noted that these animals reproduced, the resulting young constituting the third generation. The majority of the third generation young have been reared.

The young produced on these various levels of yeast (from 2 to 8 per cent of air-dried yeast and from 4 to 12 per cent of undried yeast) have been normal in every way. Repeated experiments have demonstrated the fact that when the young were placed with mothers on a good breeding ration the young were reared, whereas when the young from mothers on the breeding ration were given to the yeast-fed mothers the young succumbed. There can be no doubt that the mothers on these various yeast rations were secreting milk, otherwise it is difficult to understand how the young

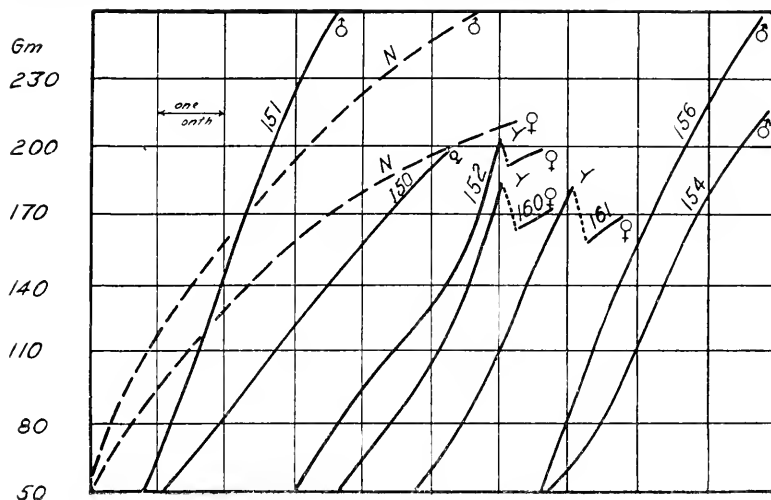


CHART 2. Curves of growth and reproduction records of second generation animals receiving 5 per cent of air-dried yeast in the diet as the sole source of vitamin B. Three females have had young. The third generation, produced by Females 160 and 161, has been reared.

could live as long as 4 or 5 weeks before they died. As a general rule the young developed an emaciated condition and died in a period of time varying from 1 to 4 weeks.

The rations employed in these experiments differ from those used by Evans and Bishop (3) primarily in the fat quota. We are inclined to believe that there is an optimum concentration of fat tolerated by the animal body, and that if this optimum concentration is exceeded sterility results. We have fed four rats on a diet consisting of casein 18 per cent, filtered butter fat 5 per cent, lard 10 per cent, salt mixture 185, 3.7 per cent, air-dried

yeast 3.5 per cent, and dextrin to 100 per cent. Although the animals have grown better than the normal rate, not one of the three females, now 7 months old, has reproduced on this ration. Results similar to those obtained with yeast have been secured with wheat embryo as the sole source of vitamin B. We expect to report later on the relation of salts, fat, and wheat embryo on growth, and especially on reproduction.

It has been observed repeatedly with a large number of animals that when salt mixture 185 (4) is employed in these rations to the extent of 5 per cent, the animals are almost without exception sterile although they grow at the normal rate to maturity. Future work will determine whether the failure of the young to grow on these various levels of yeast is to be ascribed to the difference in vitamin B necessary for growth and that required for milk secretion and normal milk composition, or whether another unknown dietary factor must be taken into account. Evidence accumulated in this laboratory indicates that it is not alone a question of the level of vitamin B. Even upon 8 per cent air-dried yeast all the young were not reared, and this is four times the amount necessary for normal growth to maturity.

Value of Yeast Proteins for Growth and Reproduction.

Data in the literature are conflicting concerning the dietary properties of yeast proteins. Funk, Lyle, and McCaskey (5) state that a large part of yeast nitrogen has no food value. They state, furthermore, that young rats can live on yeast as the sole source of nitrogen for a long while, although it has not been proved that they can do so indefinitely. Osborne and Mendel (6) found that yeast proteins, at certain levels, furnish all of the amino-acids necessary for growth. Their data concerning the value of these diets for reproduction were not conclusive, however. Some of the animals proved to be sterile. In other experiments in which yeast had been used as a sole source of vitamin B they also noted that the animals were sterile, but due to the fact that two of the four males fed yeast as the sole source of protein were fertile, they drew the conclusion that it was not the yeast *per se* which caused the sterility.

Air-dried *Saccharomyces cerevisiae* (Fleischmann's Race F) contains 45 per cent of crude protein. The following amounts of

air-dried yeast expressed in percentage were fed in these experiments: 25, 30, 35, 40, 45, and 50. These figures correspond, respectively, to the following amounts of crude yeast protein in the diet, expressed in percentage: 11.50, 13.80, 16.10, 18.40, 20.70, and 23.00. Account must be taken of the fact that a considerable quantity of the nitrogen of yeast is present in forms other than protein, but how much of the nitrogen is so represented is not known. In order to economize space only two charts are given.

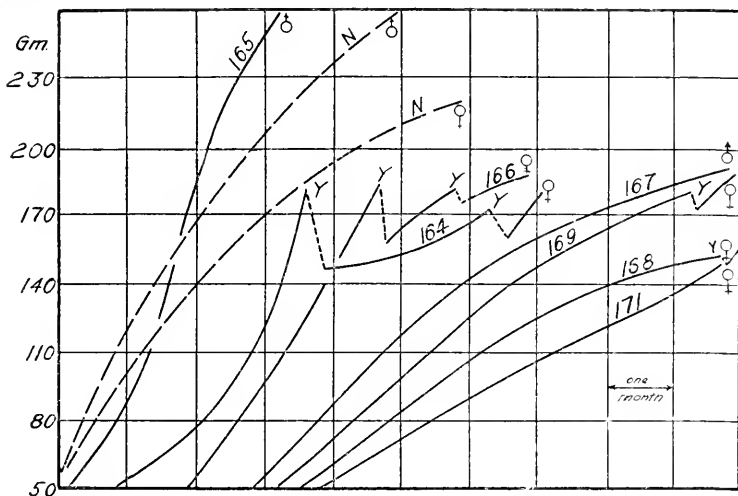


CHART 3. Typical curves of growth and reproduction records of animals receiving 45 per cent of yeast as the sole source of protein in the diet. This amount of yeast corresponds to 20.70 per cent of crude yeast proteins.

Chart 3 shows the type of growth curve obtained when 45 per cent of air-dried yeast is employed as the only source of protein. The ration consisted of air-dried *Saccharomyces cerevisiae* 45 per cent, corresponding to 20.70 per cent of crude yeast proteins, sodium chloride 1 per cent, calcium carbonate 1 per cent, filtered butter fat 5 per cent, and the remainder of the ration to 100 per cent was composed of dextrin. A large number of experiments performed in this laboratory demonstrate conclusively that animals are not sterile on this amount of yeast intake, and that the proteins of yeast at this level furnish all of the amino-acids necessary for growth and reproduction. Furthermore, sodium chloride

and calcium carbonate are the only inorganic constituents that it is necessary to add in order to obtain normal growth. In this respect yeast is similar to seeds which are known to be too low for normal growth and well being in the three ions, calcium, sodium, and chlorine. Animals 167, 168, 169, and 171 are young which were produced by Females 164 and 166. The curves of growth of the young are below normal. The young grew slowly for a considerable period of time shortly after they were weaned. This part of the growth curve is not shown in the chart. In spite of the fact that the curves of growth are not normal, the chart

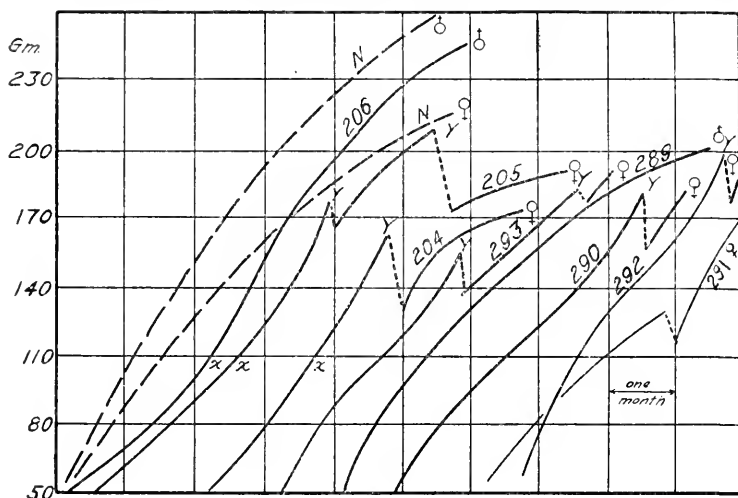


CHART 4. Curves of growth of rats receiving 30 and 25 per cent of yeast in the diet as the sole source of protein. Animals 204, 205, and 206 received 30 per cent and the others, 25 per cent of yeast.

shows that two of the second generation animals have thus far had young which likewise have grown below the normal rate. Third generation animals have been reared not only on a level of 45 per cent of yeast but also on 40, 35, and 30 per cent of yeast intake. In practically all cases the second and third generation animals have grown below the normal rate, although the second generation young have almost without exception proved fertile.

Chart 4 shows typical curves of growth obtained on rations containing 30 and 25 per cent of yeast as the sole source of protein. Animals 204, 205, and 206 received the following ration: yeast

30 per cent, sodium chloride 1 per cent, calcium carbonate 1 per cent, filtered butter fat 5 per cent, and dextrin 63 per cent. At the point marked x the sodium chloride and calcium carbonate were substituted for a salt mixture equivalent to 3.0 per cent. Growth and reproduction were normal. Third generation animals have been reared on this level of yeast, although the second and third generation animals grew below the normal rate, especially during and shortly after the weaning period. Rats 289, 290, 291, 292, and 293 received a ration consisting of yeast 25 per cent, filtered butter fat 5 per cent, sodium chloride 1 per cent, calcium carbonate 1 per cent, and dextrin to 100 per cent. Growth is normal in the first generation, and the young are produced at normal intervals, but the young do not grow at the normal rate. At 11 weeks of age the surviving individuals weigh only about 50 to 60 gm. each. Whether the failure of the young to grow normally is due to an inadequacy of the ash constituents, an insufficiency of amino-acids, or to both of these factors, it is difficult to say. Animals on 50 per cent of yeast as the sole source of protein grow normally for about 3 to 4 months when the curves of growth flatten out. At this level of intake yeast is apparently toxic. Just as in the preceding experiments on yeast as a source of vitamin B, it has been conclusively demonstrated, on a large number of animals, that when these various levels of yeast are fed, together with 5 per cent of salt mixture 185 (4) the animals are almost without exception sterile.

The writers desire to thank The Fleischmann Company for supplying the yeast employed in these experiments.

SUMMARY.

1. The postulation of a new vitamin for reproduction is unnecessary. Third generation animals have been reared on 5 per cent of yeast as the sole source of vitamin B in the diet.

2. The majority of the young are not reared on synthetic diets containing yeast as the only source of vitamin B.

3. When 5 per cent of salt mixture 185 is employed in the diet, the majority of the animals are sterile.

4. Yeast proteins are good proteins. Third generation animals have been obtained on 45, 40, 35, and 30 per cent of yeast in the diet as the sole source of protein.

5. On 25 per cent of yeast as the sole source of protein the young grow far below the normal rate.

6. There is nothing of a toxic nature in yeast to account for the failure of the young to develop when yeast is employed as the sole source of vitamin B.

7. In order to make the ash constituents of yeast complete, only the inorganic ions, calcium, sodium, and chlorine need be added.

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THE ACTION OF PROTEOLYTIC ENZYMES UPON INSULIN.

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Insulin is the name applied to a solution obtained from the pancreas of animals which has the power of increasing the combustion of carbohydrates in diabetes. Just what this solution contains that has this property is by no means entirely known. In this paper results obtained by the action of commercial proteolytic enzymes on insulin are described. The results obtained indicate that insulin is protein-like in nature.

Protein Nature of Insulin.

Insulin preparations obtained by the Collip method show the typical qualitative reactions of proteins. This means, if insulin itself is not a protein or polypeptide, that it is contaminated with alcohol-soluble protein. The simplest and most obvious way to determine whether the substance, having the properties of insulin, is thus contaminated with protein is to subject the preparation to the action of proteolytic enzymes¹ by which the presumably contaminating proteins would be removed by hydrolysis. If the solutions still showed activity it would prove that the protein was merely an impurity.

A series of experiments were done with trypsin, papain, and pepsin, using Lilly and Co.'sletin and our own insulin (prepared by Collip's method²).

¹ Banting and Best (Banting, F. G., and Best, C. H., *J. Lab. and Clin. Med.*, 1921-22, vii, 251) state that pancreatic juice destroys the activity of the liquid pressed off of macerated degenerated dog pancreas. This is the only statement concerning the behavior of insulin preparations toward proteolytic enzymes that we have been able to find in the literature.

² Until about Mar. 15, 1923, our own insulin was all required for clinical use, but we were sometimes given certain Lilly and Co. preparations for chemical experimentation. Since that date we have received no such preparations and have used our own.

Action of Trypsin on Insulin.—7.5 cc. of a Lilly and Co. preparation of iletin (No. 67569.725708) containing 40 units in 5 cc. (label) were diluted with an equal volume of water. Of this 5 cc. were placed in a flask as Experiment 1. The remaining distinctly acid solution was neutralized to litmus with NaHCO_3 and a small excess added. This gave a solution having the optimum reaction for peptone hydrolysis with trypsin; *i.e.*, the reaction of a NaHCO_3 solution containing free CO_2 . Half of this solution constituted Experiment 2. To the remainder 0.01 gm. of trypsin (powder) was added. After standing 42 hours at room temperature 1 cc. of these solutions was injected into three rabbits (approximately 2 kg.) that had been fasted for 18 hours.

First Series.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		<i>per cent</i>	<i>per cent</i>	
1	5 cc. diluted iletin.....	0.13	0.043	After 1.5 hrs. (in convulsions).
2	5 “ “ “ + NaHCO_3 ...	0.15	0.057	After 2 hrs. (no convulsions).
3	5 “ “ “ + “ + 0.01 gm. trypsin.....	0.14	0.13	After 2 hrs. (no convulsions).

Second Series.—The above experiments were repeated with our own preparation of insulin. 0.03 gm. of trypsin was suspended in 7.5 cc. of water. 6.0 cc. of insulin (No. 45, of which 0.3 cc. produced pronounced convulsions in less than 2 hours) were diluted with 4 cc. of water and treated with a slight excess of NaHCO_3 .

The experiments were set up as stated in the table and after 42 hours 1 cc. portions of the solutions were injected into ± 2 kg. fasted rabbits as before.

Second Series.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		<i>per cent</i>	<i>per cent</i>	
1	1.5 cc. insulin No. 45 + 3.5 cc. water.....	0.13	0.03	After 1.25 hrs. (in convulsions).

Second Series—Continued.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
2	2.5 cc. insulin + NaHCO_3 + 2.5 cc. water	0.12	0.023	After 1.25 hrs. (in convulsions).
3	2.5 cc. insulin + NaHCO_3 + 2.5 cc. trypsin solution.....	0.12	0.13	After 2 hrs. (no convulsions).
4	2.5 cc. insulin + NaHCO_3 + 2.5 cc. trypsin solution (boiled 5 min. before adding to destroy the enzyme).....	0.14	0.037	After 2 hrs. (no convulsions).
5	2.5 cc. trypsin solution + 2.5 cc. water.....	0.15	0.14	After 2 hrs. (no convulsions).

Third Series.—The experiments in the second series were repeated using our own insulin (No. 52) of which 0.2 cc. produced convulsions in less than 2 hours.

Third Series.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		<i>per cent</i>	<i>per cent</i>	
1	1.5 cc. insulin No. 52 + 3.5 cc. water.....	0.10	0.03	After 1.25 hrs. (in convulsions).
	2.5 cc. insulin + NaHCO_3 + 2.5 cc. water	0.10	0.03	After 45 min. (died in con- vulsions).
3	2.5 cc. insulin + NaHCO_3 + 2.5 cc. trypsin solution.....	0.10	0.10	After 2 hrs. (no convulsions).
4	2.5 cc. insulin + NaHCO_3 + 2.5 cc. trypsin solution (heated 12 min. in boiling water to destroy the enzyme).....	0.10	0.03	After 2 hrs. (no convulsions).
5	2.5 cc. trypsin solution + 2.5 cc. water.....	0.10	0.11	After 2 hrs. (no convulsions).

Action of Papain on Iletin.—Similar experiments were made using the same Lilly and Co. preparation as before and commercial papain as the proteolytic enzyme. 7.5 cc. of iletin were diluted

with 7.5 cc. of water and treated with a slight excess of NaHCO_3 since Oppenheimer³ states that papain works best in nearly neutral solution.

After standing 45 hours at room temperature 1 cc. portions of the solutions were injected into ± 2 kg. fasted rabbits as usual.

Papain Experiments.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		<i>per cent per cent</i>		
1	5 cc. diluted iletin.....	0.11	0.025	After 1.5 hrs. (in convulsions).
2	5 " " " + NaHCO_3 ...	0.12	0.030	After 1.25 hrs. (in convulsions).
3	5 " " " + " + 0.01 gm. papain.....	0.125	0.125	After 2 hrs. (no convulsions).
4	5 cc. water + 0.01 gm. papain...	0.135	0.135	After 2 hrs. (no convulsions).

Action of Pepsin on Iletin.—The same Lilly and Co. preparation was tested with commercial pepsin. 7.5 cc. of iletin were neutralized with a slight excess of NaHCO_3 . 0.3 gm. of pepsin were suspended in 0.2 per cent hydrochloric acid solution.

After 45 hours 1 cc. portions of the solutions were injected into ± 2 kg. fasted rabbits as before.

Pepsin Experiments.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		<i>per cent per cent</i>		
1	2.5 cc. iletin + 2.4 cc. water	0.11	0.025	After 1.5 hrs. (in convulsions).
2	2.5 cc. iletin + NaHCO_3 + 2.5 cc. water.....	0.14	0.024	After 1 hr. (in convulsions).
3	2.5 cc. iletin + NaHCO_3 + 2.5 cc. pepsin solution.....	0.13	0.10	After 2 hrs. (no convulsions).
4	2.5 cc. iletin + NaHCO_3 + 2.5 cc. pepsin solution (boiled 5 min. to destroy enzyme).....	0.13	0.03	After 2 hrs. (no convulsions).
5	2.5 cc. pepsin solution + 2.5 cc. water.....	0.13	0.15	After 2 hrs. (no convulsions).

Interpretative Experiments.—The above experiments indicate that insulin is protein-like in nature. Before definitely concluding that this is true it was desired to criticize this result experimentally as completely as possible.

It was found in other experiments that 0.5 N NH_4OH inactivates insulin after 6 days at room temperature and that its activity is restored at once after slightly acidifying the solution with hydrochloric acid.⁴ The recent work of von Euler and Myrbäck⁵ and von Euler and Svanberg⁶ showed that saccharase is reversibly "poisoned" by *p*-phenylenediamine, *p*-toluidine, aniline, and phenylhydrazine. Moreover, the activities of certain proteolytic enzymes are decreased by the presence of products of digestion.⁷ It was therefore thought possible that insulin was inactivated in the above experiments by the digestion products of foreign protein present in its solutions.

Influence of Digestion Products on Insulin.—1.5 cc. of insulin (No. 45) + 3.5 cc. of water were treated with 0.01 gm. of trypsin at room temperature for 2 days. The flask was then immersed in a boiling water bath for 10 minutes. After cooling 1.5 cc. of insulin (No. 45) were added. The next day 1.3 cc. of the mixture were injected into a fasted 2 kg. rabbit. The rabbit developed convulsions in 2 hours. The blood sugar was 0.13 per cent before injection and 0.04 per cent 2 hours later. The dose as given was equivalent to 0.3 cc. of the undiluted insulin (No. 45) which always produced convulsions in about 2 hours and reduced the blood sugar to 0.02 to 0.04 per cent in 2 hours. This experiment shows that insulin is neither neutralized nor inactivated by the chemical action of the products of tryptic digestion of the proteins present in insulin prepared from beef pancreas. These results do not give any basis for modifying the conclusion that insulin is protein-like in nature.

Formol Titration of a Trypsin Digestion of Insulin.—If it could be shown that the "digestion" of insulin does not increase the formol titration of the solution it would indicate that the influence

⁴ These observations will be described in full in a paper now in preparation.

⁵ von Euler, H., and Myrbäck, K., *Z. physiol. Chem.*, 1923, exxv, 297.

⁶ von Euler, H., and Svanberg, O., *Fermentforsch.*, 1920-21, iv, 29, 54.

⁷ Oppenheimer,³ p. 78.

of proteolytic enzymes was some chemical effect other than digestion.

The experiments were set up as follows:

No.	Material used.
1	7 cc. trypsin solution (0.025 gm. of trypsin) + 3 cc. water.
2	7 " " " (0.025 " " ") + 3 " insulin No. 45.
3	3 cc. insulin No. 45 + 7 cc. water.

After standing 2 days at room temperature these solutions were titrated by the usual formol titration method. When the results for Nos. 1 and 3 were added together and compared with those for digested insulin in No. 2 it was evident that the amino-acid content of No. 2 was increased corresponding to 1.73 cc. of 0.1 N NaOH; *i.e.*, about 50 per cent.

This shows that the digestion of insulin solution gives rise to titrable amino-acids, but of course this experiment alone does not indicate whether they were derived from protein impurities or from insulin.

Does the Protein Serve as a Colloid Protector?—It has been suggested that the protein in enzyme preparations acts as a colloid protector. This idea rests on such observations as those of Paal, who found that colloidal palladium suspended in proteins is rendered inactive as a catalyst for hydrogenation, when the protein is digested with enzymes. This did not occur when the protein was replaced by protalbic and lysalbic acids, which are not much digested by proteolytic enzymes. Waentig and Steche,³ who destroyed catalase with trypsin, considered at first that possibly the protein colloid protector only had been digested. On the basis of later and exhaustive studies on catalase Waentig and Gierisch⁹ concluded that this reservation does not apply, that catalase is really a protein and that its inactivation by proteolytic enzymes is not due to the destruction of a protective colloid.

Therefore, this suggestion can certainly not stand against the protein-like nature of insulin until some tangible basis for its support is discovered.

³ Waentig, P., and Steche, O., *Z. physiol. Chem.*, 1913, lxxxiii, 315.

⁹ Waentig, P., and Gierisch, W., *Fermentforsch.*, 1914-16, i, 165.

Discussion of Results Obtained with Proteolytic Enzymes.—The above results with trypsin, papain, and pepsin prove that these enzymes destroy the activity of insulin preparations. That this is not a simple neutralization is indicated by the fact that boiled trypsin and pepsin do not change the activity of insulin even on standing in contact with it for 42 hours and by the fact that digested insulin does not inactivate undigested insulin. The simplest conclusion from these results is that insulin itself is protein-like in nature.

Such a conclusion is in harmony with the existing knowledge concerning the best known enzymes, and there is no *a priori* theoretical reason for doubting its truth. In fact, since enzymes must be elaborated and manipulated within protoplasmic systems, it seems simple and logical to assume that they will have solubility relations well suited to this medium. All in all, similarity in constitution is one of the safest guarantees of solubility now known. We could, therefore, reasonably expect compounds, so indispensably related to protoplasmic activity as enzymes are, to have definite structural relationships to the proteins in their constitution. Such an arrangement would help to simplify the problem of organizing vital processes as it exists in higher organisms. Thus the unspecialized proteins of the cell would constitute the raw material from which enzymes could be elaborated at once when needed.

On the other hand, the idea that enzymes may generally be protein-like does not mean that they are not strikingly different in some respects from the rank and file of proteins.

The conclusion that insulin is protein-like in nature is contradicted by the results of Best and Macleod,¹⁰ who isolated from fish (skate) an insulin preparation that does not give the color reactions for protein. They also state the following: "That the trace of protein in ox insulin preparations is an impurity is evidenced by the fact that after precipitation with phosphotungstic acid and removal of the latter by ether and barium the preparation retains its powers to cause hypoglycemia." On the other hand, all their preparations from ox pancreas gave the color reactions for proteins. The solid insulin preparation described by Doisy,

¹⁰ Best, C. H., and Macleod, J. J. R., *J. Biol. Chem.*, 1923, lv, p. xxix.

Somogyi, and Shaffer¹¹ contained 14 per cent nitrogen and showed a high activity. If this material which separates at the isoelectric point is all insulin it is undoubtedly a protein.

Three Types of Insulin Preparations.

On the basis of our present knowledge there are at least three distinct types of insulin preparations.

1. *The Protein Type.*—This is the preparation of Doisy, Somogyi, and Shaffer.¹¹ We have not prepared insulin by their method, but we have regularly obtained a protein precipitate from our strong final aqueous solution. After precipitating the insulin with alcohol the extract was taken up in water and passed through a Berkefeld filter. On standing in the refrigerator a fine white "insulin" powder separated during the first few days. One such precipitate, after being washed with 80 cc. of distilled water in two portions, was suspended in 10 cc. of distilled water. Of this suspension 1 cc. was injected into a 2 kg. fasted rabbit. The blood sugar before injection was 0.113 per cent and 0.02 per cent 2 hours after injection; the rabbit was found in convulsions after 2 hours. This precipitate was afterwards dried out in a desiccator over CaCl_2 and was found to weigh 0.03 gm. This was again suspended in 10 cc. of distilled water after a month in the desiccator. On injecting 1 cc. of the suspension into a fasted rabbit convulsions appeared in 2 hours and the blood sugar dropped from 0.10 to 0.03 per cent. When the supernatant water, from which this active precipitate had been separated, was injected it produced no change in the blood sugar.

If insulin is adsorbed in this extremely fine protein powder it must be held with great avidity not to be removed by such prolonged action of so much water during the centrifuging of the fine precipitate.

The protein type of insulin differed from the two succeeding types in being insoluble in water and gave the blue-violet biuret test.

2. *The Peptone-Polypeptide Type.*—This preparation differs from the preceding in being easily soluble in water and in giving the red biuret test.

¹¹ Doisy, E. A., Somogyi, M., and Shaffer, P. A., *J. Biol. Chem.*, 1923, **lv**, p. xxxi.

The major portion of our insulin preparation does not separate as a solid. Our own insulin preparations, from which the above protein precipitate no longer separated after standing in the refrigerator for weeks, gave a violet-red biuret test, in which it was sometimes quite difficult to detect any violet tint at all. On the other hand, all Lilly and Co. preparations that we have tested gave an unmistakably violet colored biuret test. The colors were compared with the biuret test obtained with peptone (Chassaing, Paris), which gives a reddish violet tint and with peptone (Armour) which gives a bluish violet tint. Lilly and Co.'s iletin preparations gave colors resembling that of the peptone (Armour).

From the above tests we have concluded that the soluble insulin preparations that we have seen belong to this peptone-polypeptide type.

3. The Abiuret Type.—This type of insulin may be present as one of the components in the preceding type, but it deserves separate consideration because of the relative simplicity of its constitution. As quoted above, Best and Macleod obtained insulin from skate and from beef pancreas (by a special treatment) that gives neither the biuret test nor the qualitative reactions of protein. We have not investigated this type of insulin. It is obvious, however, that the synthesis of insulin will most likely be accomplished first with this type.

We do not wish to conclude too much from these tests, but there is certainly diversity enough in the behavior of the above preparations in the biuret test and in the solubility in water, even in those observed by us, to permit of concluding that insulin is a protein, a peptone, or a polypeptide,¹² depending upon which preparation was tested.

This diversity of characteristics and properties of insulin may seem surprising at first, but is readily accounted for when we consider that insulin constitutes the "spoils" obtained by a certain process of "sifting" the "debris" of pancreas tissue broken down mechanically as much as possible. The "size" of the pieces obtained will depend on all the conditions involved in the material used and on the nature of the "sieve" or process of preparation used.

¹² Robertson (Robertson, T. B., *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, 1918, 14) states that many polypeptides including tripeptides give typical biuret reactions.

If it is granted that the action of the proteolytic enzymes on insulin in the experiments described above was a true proteolytic hydrolysis, involving the breaking up of at least one amino-acid coupling and, therefore, presumably containing at least one amino-acid in its molecule, then the known facts will admit of two simple interpretations.

1. Insulin is a simple polypeptide that does not give a biuret reaction nor any of the typical protein reactions. It is, however, readily adsorbed by more complex protein derivatives and is brought down by them as in the "solid insulin" of Doisy, Somogyi, and Shaffer, and in various processes of precipitating insulin.

2. Insulin is a group or radical present in certain proteins which is easily hydrolyzed by proteolytic enzymes. This radical may occur in protein compounds of greater or less complexity and, therefore, its qualitative reactions will range all the way from a negative biuret, for the simplest group that still retains the characteristic properties of insulin, to those of a typical protein.

The second of the above interpretations includes the first in a different form. The first interpretation will be more attractive to those interested in eventually manufacturing insulin synthetically and in developing clearer views of the mechanics of its action, in as far as its effects can be ascribed to a given chemical constitution. The second interpretation conforms better with most of the things that we know about life and chemistry and is more likely to be correct because it is more general in its outlines. For instance, it permits us to consider that insulin is a chemical effect, that probably all living organisms have an insulin function, that the relative ease of isolation of the active grouping will vary from one organism to the other, that any undesirable secondary clinical effects may be controlled by preparations from other sources, that there is more than one chemical configuration possessing the insulin effect, and other interesting propositions.

If insulin is considered to be a specific chemical substance, the conclusion that it may be a polypeptide, peptone, or protein, depending upon the process used and the portion of the product tested, is unsatisfactory. If, however, the insulin effect is conceived of as associated with a certain group or grouping, the above interpretation indicates that this group persists in an effective form in several stages of protein breakdown. If this is true, it

may be found, when the facts are known, that the insulin effect will parallel the lipase effect, which is found under suitable conditions even with certain amino-acids as well as polypeptides and proteins,¹³ but is, of course, best developed in lipase preparations. These experiments indicate that the parallelism cannot go down so far as the free amino-acids since the digestion products of insulin were found to have no insulin activity.

SUMMARY.

The fact that insulin preparations are completely inactivated by proteolytic enzymes (trypsin, papain, pepsin) indicates that the compounds present in the preparation, that give the qualitative reactions for proteins, are not all impurities, and that insulin itself is protein-like in nature. Attempts to break down this conclusion by other interpretative experiments have so far failed.

In discussing the available information upon the nature of insulin it is suggested that insulin is the "effect" of a certain group or grouping. This "effect" appears to be associated with a protein on the one hand and peptones and polypeptides on the other.

If this interpretation of the known facts concerning insulin is correct, it does away with the contradictions concerning its nature, existing in the literature, and also conforms with the new facts presented in this paper.

¹³ Falk, K. G., *The chemistry of enzyme action*, New York, 1921, 84.

THE REFRACTOMETRIC DETERMINATION OF HEMOGLOBIN.

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INTRODUCTION.

The standard method of determining hemoglobin concentration in blood is based on a measurement of oxygen capacity and Hüfner's factor, 1.34 cc. of oxygen per gm. of hemoglobin. Unfortunately, the Van Slyke and the Haldane machines do not give the same results for the oxygen capacity. The difference is over 10 per cent of the total, and there is no agreement as to which method is correct. The colorimetric methods are based on the oxygen capacity, so that they can offer no help as to the absolute value.

The refractometer appeared to offer the possibility of making a determination of the absolute quantity of hemoglobin by an independent method, entirely unconnected with the oxygen capacity, or even with any chemical standards. There was also the possibility that a rapid and convenient method might be worked out for determining hemoglobin, which could be used by those having refractometers at their disposal.

After the work was started it was found that Howard 3 years ago¹ had investigated the refractive constant of hemoglobin and had suggested the possibility of a refractometric method for blood. No such method has been published, however, and the amount of elapsed time since that suggestion seemed to the writers to justify a new attack on the problem.

The principal objects in the present work were: (1) to redetermine the refractive constant "*a*" of hemoglobin; (2) to test the constancy of the relation $a \times c = n_{\text{solution}} - n_{\text{water}}$; (3) to

¹ Howard, F. H., *J. Biol. Chem.*, 1920, xli, 537.

devise a method for the determination of hemoglobin in blood which should first of all be accurate, and give the absolute quantity of hemoglobin in terms of dry weight of the substance; (4) to compare the concentration of hemoglobin with oxygen capacity; and (5) to devise a quicker method that could be used with convenience.

Note.—In all cases in this paper where figures are given for the refractive index of a solution minus the refractive index of water at the same temperature, it is to be understood that the last integer is in the fifth decimal place; e.g., 194 represents 0.00194.

In all the experiments the temperature was controlled by immersing the usual refractometer bath in a large (370 liter) water bath. The temperature did not vary faster than 0.1°C. per hour, and corrections were made for temperature when necessary.

A. Determination of "a" of Hemoglobin.

A redetermination of this value was deemed advisable for the following reasons: (1) Howard's values were not based on human blood with the exception of one experiment where the concentration was standardized only by the oxygen capacity. (2) The weight of hemoglobin thoroughly dried in an oven at 110°C. was considered preferable to the weight of crystalline hemoglobin (used by Howard). Not that it is theoretically preferable, but merely that until the ease of loss of water of crystallization and the possibility of more than one degree of hydration of the crystals are carefully investigated, it seems probable that a more constant value will be obtained from a thoroughly dried preparation. The writers do not believe their method of drying is unexceptionable. It ought, however, to give minimal values, and this is especially important in view of our later findings with relation to the oxygen capacity.

1. Preparation of the Hemoglobin.—The hemoglobin was prepared from human blood by the method of Adair. This essentially consists in laking the washed red cells with ether, adding salt to separate the stromata, and dialyzing the hemoglobin solution. All operations are done at 0°C.

Bloods from two normal human individuals were investigated.

Instead of measuring the hemoglobin solution taken for the dry weight volumetrically, it was considered more accurate to get the specific gravity of the blood, and then weigh the sample taken.

2. Determination of the Concentrations of the Solutions in terms of Dry Weight.

Specimen 1.

Density (by 5 cc. pycnometer) at 19.5°C.

Weights..... (1) 5.1838 gm.

(2) 5.1854 "

Average..... 5.1846 "

Capacity of pycnometer..... 4.9916 cc.

Density..... 1.03386

Hemoglobin solution for dry weight (weighed in crucible)..... 5.1942 gm.

Hemoglobin solution for dry weight in cc., calculated from density..... 5.001 cc.

Dry weight (dried at 110°C. for 3 days)..... 0.8173 gm.

Concentration of original solution..... 16.34 per cent.

The ash was 0.0044 gm. The ash calculated from the hemoglobin was 0.0040 gm.

Specimen 2.

Density at 20.5°C.

Weight..... 5.2353 gm.

Capacity of pycnometer..... 4.9916 cc.

Density..... 1.0488

	1	2
Hemoglobin solution for dry weight (weighed in crucible).....	5.2282 gm.	5.2044 gm.
Hemoglobin solution for dry weight in cc., calculated from density.....	4.9885 cc.	4.9624 cc.
Dry weight (dried to constant weight).....	0.9993 gm.	0.9916 gm.
Concentration of original solution.....	20.048 per cent.	19.988 per cent.
Ash found.....	0.0064 gm.	0.0068 gm.
" calculated.....	0.0050 "	0.0050 "

3. Determination of the "n" of the Solution and of the Value of "a."

In each case the refractive index of water at the same temperature as the solution is subtracted from the refractive index of the

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solution. The pipettes were calibrated, and the factors omitted in the statement of the dilutions, but, of course, used in the calculations.

Specimen 1.

Dilutions.	$\frac{n \text{ Solution} - n \text{ water}}{\text{Dilution}}$	Remarks.
1:25	3, 181	The maximum variation in the values is 3,185-3,162 = 23. This is equivalent to $23 \div 194.4 = 0.12$ per cent Hb.
2:25	3, 185	
1:2	3, 162	
Average.	3, 177	

$$a = \frac{n \text{ Solution} - n \text{ water}}{\text{Concentration}} = \frac{3,177}{16.34} = 194.4$$

Specimen 2.

Dilutions.	$\frac{n \text{ Solution} - n \text{ water}}{\text{Dilution}}$
1:4	3, 883
1:8	3, 908
1:16	3, 904
1:25	3, 851
Average.....	3, 886.5

$$a = \frac{n \text{ Solution} - n \text{ water}}{\text{Concentration}} = \frac{(1) \ 3886.5}{20.048} = 193.85$$

$$(2) \ \frac{3886.5}{19.988} = 194.45$$

$$\text{Average.....} \ 194.15$$

The average of the three values of the factor is 194.2. Using the weighted average method of Robertson,² *i.e.* dividing the sum of the original refractive indices by the sum of the dilutions, the second specimen gives the average of 194.17. The maximum variation in the factor calculated in the various instances is 194.45-193.85 = 0.6 or 0.3 per cent of the original factor. Applying the factor to hemoglobin concentration in blood would give an uncertainty due to the factor of 0.3 per cent of the total hemoglobin, or if the hemoglobin is 15 per cent, of 0.045 per cent of hemoglobin. This is certainly accurate enough for all practical purposes.

² Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179.

The maximum variation in the factors from our average factor is $194.2-193.85 = 0.35$ or 0.2 per cent of the total. This would represent, applied to blood, an uncertainty of 0.030 per cent of hemoglobin.

The difference from Howard's factor of 183 we think is to be explained by the fact that his preparation contained water of crystallization. If so, there would be $\frac{194.2-183}{194.2} = 5.7$ per cent of water of crystallization. Gamgee (quoted in Schafer's Text-book of physiology) found the hemoglobins of various animals to contain from 4 to 8 per cent of water. The crystals were dried *in vacuo* at low temperature, and then at 110°C .

B. Determination of the Constancy of the Relation $a \times c = n$ Solution — n Water.

The best way to determine whether this relation is valid is to calculate the value for the more dilute solutions from the value for the more concentrated, and note whether the observed value for the diluted solutions differs from the calculated value by more than the errors of observation.

Proportionality of the Refractive Index of Hemoglobin.

Specimen No.	Dilution.	Concentration from which calculated.	Calculated	Found.	Difference.
1	2:25	1:2	256.7	258.6	1.9
1	1:25	1:2	128.4	129.0	0.6
1 (new dilution).	1:2	Original solution, a dilution of 1, having an index of 62.	31.0	29.4	1.6
1 (" ")	1:4	" " "	15.5	15.3	0.2
1 (" ")	1:8	" " "	7.8	7.7	0.1
2	1:8	1:4	485.7	487.7	2.0
2	1:16	1:4	242.8	244.0	1.2
2	1:16	1:8	243.9	244.0	0.1
2	1:25	1:8	155.0	156.0	1.0

The error of observation might be as high as 3. All deviations are within this value. There is therefore proportionality in the range covered, which is that from an 8.17 per cent solution to a 0.04 per cent solution. More concentrated solutions than 8.17

per cent are too difficult to read. This conclusion is in complete accord with the observations of Howard on hemoglobin and of Robertson on various other protein solutions.

C. A Refractometric Method for Hemoglobin in Blood.

1. *The Method.*—The method in brief is: (1) Wash the red cells from a measured quantity of blood. (2) Hemolyze with distilled water and saponin, add salt to throw down the stromata, and make up to volume. Centrifuge. (3) Obtain the refractive index of the solution. (4) Obtain the refractive index of the filtrate obtained by the heat coagulation of the hemoglobin. (5) Subtract (4) from (3) and divide by “*a*” for hemoglobin.

Thus the method is essentially a separation of the refractive index of hemoglobin from the refractive indices of the other blood constituents. After the proof which follows of the admissability of the various procedures, will be given a detailed statement of the steps in the method.

2. *Separation from Plasma.*—The amount of necessary washing with 0.8 per cent NaCl was determined repeatedly by getting the refractive index of the wash water and comparing it with that of the NaCl. The washings were done on 2 cc. of blood in a 16 to 20 cc. centrifuge tube. To the blood was added 0.8 per cent NaCl up to 15 cc. The mixture was centrifuged 5 minutes at low speed and the diluted plasma pipetted off. NaCl solution was then added as before, the tube inverted with a rubber stopper in, and thoroughly shaken, then again centrifuged. Invariably the third wash water (counting the original dilution of the blood as the first) was not appreciably different from the NaCl solution. That this is reasonable is evident from the following calculation. If 1.2 cc. of plasma are present in the blood, the first dilution gives a plasma concentration of approximately $1.2 \div 13.8 = 0.087$. The next wash gives, if 0.2 cc. diluted plasma is left with the red cells, $0.087 \div 71 = 0.0012$, and the third, $0.0012 \div 71 = 0.0000172$. If the refractive index of the plasma — that of water were 1,650, then that due to the traces of plasma in the third dilution would be $1,650 \times 0.0000172 = 0.028$. A difference of 2.0 is all that can be read with any certainty. Of course there are traces of plasma protein that would give protein tests such as the nitric acid test,

but they have no significance in this connection. In the method the cells after washing are made up to 10 cc. This final dilution would then correspond to the third washing above, and only two washings are necessary.

3. *Methods of Hemolysis.*—Hemolysis with distilled water alone was found to be incomplete under the conditions of this method. That is, using 2 cc. of blood, the washed red cells have about 7 cc. of distilled water added, then 1 cc. of 10 per cent NaCl, and then, after making up to 10 cc., are centrifuged out. The stromata appeared deep red, and careful determinations of the hemoglobin concentration in the stromata by repeated extractions, showed that it was about twice as great in the stromata as in the fluid surrounding them.

With saponin hemolysis there is hemoglobin in the solution left between the centrifugalized stromata, and also hemoglobin in solution in the stromata. Therefore, the solution cannot be pipetted from the stromata and made up to volume. If, on the other hand, the stromata are left in while the solution is made up to volume, the question is important whether the concentration of hemoglobin in the stromata is the same as that in the solution surrounding them. To settle this point a considerable number of duplicate determinations were made on 2 cc. samples of blood. In one case to the washed red cells were added about 7 cc. of distilled water and about 1 mg. of saponin. Then, after thorough mixing, the solution was made up to 10 cc. with distilled water and 1 cc. of 10 per cent NaCl. The solution was centrifuged and the refractive index read. The hemoglobin was then determined from the heat filtrate according to the method described later.

In the other case, the washed red cells were hemolyzed as in the first example, and then 1 cc. of 10 per cent NaCl was added and the solution centrifuged. The solution was then pipetted off and the stromata repeatedly hemolyzed and precipitated in the same manner until they were perfectly white and the extract was colorless. The hemoglobin was then determined in the combined extracts. In the course of the extractions, especially after the first two or three, the stromata tended to dissolve. In order to be sure that they were not adding to the refractive index of the solution, the hemoglobin in these later extracts was also determined

colorimetrically, using as a standard one of the first extracts which had no dissolved stromata. Since the stromata are colorless, any increase in the refractive index should throw the determination of hemoglobin by this method out of harmony with that determined colorimetrically. No difference, not within the errors of reading the colorimeter, was found. If anything, the colorimetric figures were higher, probably on account of the turbidity of the solution. In fact, the difficulties in the colorimetric work from this cause made very exact determinations impossible. All that could be concluded was that either the stromata added very little to the refractive index, or else that they went through into the heat filtrate and were deducted.

*Concentration of Hemoglobin in Stromata Laked with Distilled Water and Saponin.**

Specimen No.	A Leaving stromata in the solution.	B Total hemoglobin, extracting stromata.	Remarks.
1	2, 873	2, 852	Slight loss in pipetting A.
2	2, 849	2, 866	
3	2, 798	2, 812	
4	3, 130	3, 123	Here much saponin was used in A.
5	2, 816	2, 836	
Average.	2, 893	2, 898	

* Concentration is given in terms of refractive index excess over that of water at the same temperature.

It is evident that there is no constant or significant difference between the two methods. The divergencies are probably due to the numerous manipulations in the repeated extractions. It appears, therefore, safe to rely upon the concentration of hemoglobin within the stromata being essentially the same as in the surrounding fluid when the red cells are hemolyzed with distilled water and saponin under the conditions of these experiments.

4. *Possible Effects of Saponin on the Refractive Index.*—In this method about 1 mg. of saponin is used to 10 cc. of solution. A solution of 70 mg. of saponin in 100 cc. of salt solution increases the refractive index 10.5. 1 mg. in 10 cc. therefore would increase

it only $10.5 \times \frac{10}{70} = 1.5$. This is within the error of reading the refractometer, and would correspond to an amount of hemoglobin in the original blood of $\frac{1.5 \times 5}{194.2} = 0.038$ per cent. (The "5" in the numerator allows for the dilution of the 2 cc. of blood to the 10 cc. of final solution.) Further, tests of the filtrates (obtained by heat coagulation of the hemoglobin as described later) showed that even when less than 1 mg. of saponin was used, the filtrate was hemolytic, and that the hemolytic power increased with the amount of saponin in the original solution. There seems no doubt, then, that the saponin goes through into the filtrate as do the other soluble non-coagulable constituents, and is allowed for when the heat filtrate is subtracted. Variations in the amount of saponin therefore do not introduce a source of error so far as the effect of the refractive index of saponin is concerned.

5. *Possible Effects of Solution of the Stromata.*—When about 1 mg. of saponin is used there is usually little or no solution of the stromata. Occasionally the stromata dissolve somewhat, however, without any apparent reason. With large amounts of saponin they dissolve completely. It appeared logical that if the irregular slight solution made much difference duplicate determinations would not check. Very careful experiments showed a maximum variation in duplicates of 0.15 per cent that apparently could be accounted for in no other way. The higher values always occurred when the stromata were completely dissolved. Attempts to prepare stromata free from hemoglobin and salts were unsuccessful. Although the error due to this factor is small, because only small amounts of saponin are used, it was nevertheless considered advisable to investigate the refractive index of the stromata, especially as it is an element in the explanation of the short method for hemoglobin described later.

The method adopted for the solution of this problem was to add to a series of 2 cc. samples of blood increasing amounts of saponin, and estimate the concentration of hemoglobin. The following tabulation gives the results.

Effect of Increased Amounts of Saponin with Solution of the Stromata.

Specimen No.	Amount of saponin.		Amount of saponin.		Amount of saponin.		Amount of saponin.		Amount of saponin.	
	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	Little (1 mg.).	15.74*	Much (10 mg.).	15.93†						
2	0.6	15.80‡	3.2	15.46§	8.0	16.06†	22.8	15.95†		
3	2.5	15.45§	5.4	15.64‡	25.9	16.24†				
4	1.0	12.72§	1.0	12.88‡	4.8	13.04‡				
5	1.3	12.43‡	2.6	12.30§	15.4	12.67†	26.0	12.73†		
	0.9.	12.41‡								
6	1.6	10.31*	2.8	10.23§	16.8	10.65†	25.4	10.73†		
	1.0	10.31*								

* Slight solution of the stromata.

† Complete solution of the stromata.

‡ Partial solution of the stromata.

§ No solution.

It will be noted that the percentage of hemoglobin varies with the degree of solution of the stromata rather than with the amount of saponin. Thus in Specimen 2 there was 0.34 per cent less hemoglobin in the case where 3.2 mg. of saponin were used than where 0.6 mg. was used. In Specimens 5 and 6 there were respectively 0.12 and 0.08 per cent less hemoglobin when about 2.5 mg. of saponin were used than when about 1 mg. was used. These last two experiments were done with the very greatest care, and it is believed represent the maximum errors due to the variation in solution of the stromata when amounts of saponin up to 3 mg. are used because the visible variation in stroma residue was as great as in any of the other similar experiments. When about 15 mg. of saponin are used the solution of the stromata becomes complete, or practically so. The apparent increase in hemoglobin due to this solution of the stromata, obtained by subtracting the figure in each series where there was none or slight solution of the stromata from the figure where there was the most complete solution gives:

Specimen No.	Increase in hemoglobin.
	<i>per cent</i>
1	0.19
2	0.6
3	0.79
4	0.16
5	0.43
6	0.50
Average	0.45
“ of Specimens 5 and 6	0.46

The refractive index of the stromata in a given amount of blood is equal to the refractive index of 0.45 per cent of hemoglobin. The error due to variations in solution of the stromata when not over 3 mg. of saponin are used is not over 0.15 per cent of hemoglobin.

6. *Possible Effects of Dissolved Leucocytes.*—The leucocytes apparently do not dissolve appreciably. Even if they did, and added to the refractive index as do the stromata, their effect would be of the order of magnitude of $\frac{10,000}{5,000,000} \times 9 \times 0.45 = 0.0081$ per cent in terms of hemoglobin concentration, which is negligible.

7. *Separation of Hemoglobin from Non-Protein Constituents by Heat Coagulation.*—At this point in the method the hemoglobin has been separated effectively as far as refractometer readings are concerned from the plasma proteins and the red cell proteins. There remains the separation from the non-protein constituents of the red cells, and from the NaCl added. These might be removed by dialysis, but the process is slow, and hemoglobin is apt to be lost by deposition on the membrane. Ultrafiltration was selected as the test method, and heat coagulation of the hemoglobin tried as a possible easy method, to be checked by ultrafiltration.

Numerous experiments on pure hemoglobin solutions showed that the hemoglobin would coagulate at 100°C. in 1 to 2 minutes, giving a clear filtrate, provided a certain amount of salt were present and the pH of the solution were close to the isoelectric point of 6.8. These conditions are fulfilled when washed red cells are hemolyzed with distilled water. The principle constituent here is hemoglobin, and the other constituents have comparatively little effect on the

pH. It is natural, therefore, that the pH of the solution should be approximately that of the isoelectric point of hemoglobin. Salts are present in sufficient amount, for 1 cc. of 10 per cent NaCl is added in the method. Invariably in numerous experiments coagulation of the hemolyzed red cell solution has occurred in 3 minutes immersion of the test-tube containing the hemoglobin solution in boiling water, and the filtrate has been clear and practically colorless. There are probably changes in the water or salt relations of the hemoglobin during the process of coagulation, but they are too slight to affect the refractometer, as the following parallel experiments on the heat filtrates and ultrafiltrates show.

Comparison of Ultrafiltrates with Heat Filtrates.

Specimen No.	n Heat filtrate - n water.	n Ultrafiltrate - n water.	Solution.
1	218.0	216.7	Hemolyzed red blood cells from 1 cc. blood made up to 25 cc. with NaCl solution.
2	4.0	4.5	Same as No. 1 but no NaCl.
3	4.0	6.0	" " " 1 " " "
4	98.0	95.0	" " " 1 " with less NaCl.
5	202.0	200.8	" " " 1 " " $\frac{1}{3}$ volume KH_2PO_4 and less NaCl.

The two methods do not differ by more than the error of reading the refractometer (2-3), although the filtrates represent widely varying salt content.

The only remaining question in this connection is whether the heat filtrate represents the salt concentration in the original solution. It may be stated that it does not exactly, of course, but experiments on pure hemoglobin solutions show that under the conditions of this method the correction would not amount to over 1.15 to 1.20 per cent hemoglobin in the final result. The authors do not care to present their results here as they are of more significance in other directions and will be published elsewhere.

Detailed Directions for the Method.

1. Measure 2 cc. of blood into a 17 cc. centrifuge tube, add 0.8 per cent NaCl up to about 15 cc., invert twice with rubber stopper in, rinse stopper into tube, centrifuge at moderate speed 5 minutes.

2. Pipette off the diluted plasma, replace with 0.8 per cent NaCl, shake vigorously with rubber stopper, rinse, and centrifuge as before.

3. Pipette off the supernatant fluid, add about 1 mg. of saponin, then distilled water up to 7 cc., mix thoroughly with a fine glass rod. Pipette into a 10 cc. volumetric flask, rinse the rod, pipette, and tube with 1 cc. of 10 per cent NaCl, and successive small portions of distilled water until the flask is full to the mark. Mix thoroughly. Centrifuge.

4. Obtain the refractive index of the solution.

5. Put the hemolyzed blood into a test-tube loosely stoppered with a rubber stopper, and place in boiling water for about 3 minutes. Cool, invert twice, filter through a small filter paper.

6. Obtain the refractive index of the filtrate.

7. Subtract (6) from (4), multiply by (5), and divide by 194.2. This gives the concentration of hemoglobin in terms of dry weight in gm. per 100 cc. of blood.

The temperature at which the solution and the filtrate are read should be the same within 0.15°C . or else correction must be made for the difference in temperature.

In some cases the blood may give after a time in contact with the prism a fuzzy line. This is apparently due to a deposition of material on the face of the prism, and all that is necessary is to take the prism out quickly, wipe the face with moist lens paper and then with dry, and replace it.

The dipping refractometer is the best for this work as it is more accurate.

Accuracy of the Method.—When the immersion refractometer is used the results should check within 0.1 to 0.2 per cent of hemoglobin, and the writers believe that the absolute amount is not in error by an appreciably greater amount.

The greatest sources of error are: (1) Volumetric technique; (2) temperature control; and (3) adjustment of the compensating prism. In hemoglobin solutions the border-line appears to move with adjustment of the prism, instead of merely showing colors as happens in colorless solutions. Sharpness of the line is the criterion here. No reading should be made unless the line is perfectly clear and sharp. In case adjustment is difficult wipe the prism as described above.

D. Relation of Hemoglobin Concentration to Oxygen Capacity.

Specimen No.	Oxygen capacity by Van Slyke method.	Average.	Hemoglobin by refractometer.	Average.	Ratio.
	<i>cc. O₂ per 100 cc. blood</i>		<i>gm. Hb per 100 cc. blood</i>		
1	18.7		14.79		1.264
2	18.2		14.67		1.25
3	18.2		14.41		1.26
4	18.2		14.50		1.255
5	19.95 20.14	20.04	15.53 15.58	15.55	1.29
6	19.21 19.21	19.21	15.76 15.74	15.75	1.22

Our method of drying would tend to give, if anything, low values for the hemoglobin. This would tend to make a high factor "a" and this in turn would tend to give a high ratio between hemoglobin and oxygen capacity. The ratio as determined by our method definitely supports the Van Slyke as opposed to the Haldane method for determining oxygen capacity.

E. A Short Method for the Refractometric Determination of Hemoglobin.

The principle of this method is to measure two equal samples of blood. To one is added a certain number of volumes of salt solution (0.8 per cent NaCl) and to the other is added the same number of volumes of salt solution containing a sufficient concentration of saponin to hemolyze the red cells. Subtracting the refractive indices and allowing for the refractive index of the saponin should give approximately at least the hemoglobin concentration in the diluted blood. The points of doubt were the liberation of other constituents than hemoglobin from the red cells, and other minor points. It seemed probable that these corrections would be insignificant. The method is checked for absolute amounts with the former method.

The method in detail is:

1. Measure two 1 cc. samples of blood into two medium size test-tubes.
2. To one sample add 5 cc. of 0.8 per cent NaCl.
3. To the other sample add 5 cc. of 0.8 per cent NaCl containing about 70 mg. of saponin per 100 cc.

4. Centrifuge (2) and (3) and read the clear solutions in the refractometer. Read also the two salt solutions.

Calculations.—Subtract the refractive indices of the two salt solutions.

This gives the added refraction due to the saponin. Multiply by $\frac{5}{5.6}$ to get the dilution of the saponin refraction by the added 0.6 cc. (approximately) of plasma. Subtract this saponin correction from (3) and then subtract (2) from the result. This gives the increase in refraction due to the hemoglobin. Divide by the hemoglobin factor, or 194.2, and multiply by 6 (the dilution) to get the concentration of hemoglobin in the original blood.

Of course, the refractive index of the salt and the saponin solutions need not be determined for every hemoglobin determination. The saponin tends to precipitate somewhat with age, so that it is advisable to check it up from time to time.

Attention needs to be directed to keeping the face of the prism clean as described in the other method, as there is a greater tendency here for the line to become blurred. The prism should be left in the solution about 3 minutes to allow it to attain the temperature of the solution, then quickly cleaned if necessary, which will not alter the temperature of the whole prism, then read in half a minute.

The blood should be taken with paraffin oil to avoid hemolysis, but great care should be taken not to get the oil mixed with the blood in the operation of shaking previous to measuring the samples. The best way to mix is to put the blood in a test-tube, let the oil rise to the top, pipette it off, then mix by introducing a large bored pipette so that the tip is near the bottom of the test-tube and draw the blood up and down rapidly.

It is possible that some samples of saponin may require more than 70 mg. per 100 cc. to effect hemolysis.

In the following tabulation are given the results obtained by the two methods:

Comparison of the Long and the Short Methods.

Specimen No.	Hemoglobin by long method.	Hemoglobin by short method.	Difference of average.	Remarks.
	per cent	per cent	per cent	
1	15.76	15.79	0.04	Saponin concentration in short method, 214 mg. per 100 cc.
	15.74	15.63		Saponin concentration in short method, 60 mg. per 100 cc.
2	15.74	15.95	0.14	Saponin concentration in short method, 70 mg. per 100 cc.
	15.80	15.88		Saponin concentration in short method, 480 mg. per 100 cc.
3	15.45	15.39	0.10	Saponin concentration in short method, 70 mg. per 100 cc.
		15.30		Saponin concentration in short method, 480 mg. per 100 cc.
4	12.30	12.56	0.26	Saponin concentration in short method, 70 mg. per 100 cc.
		12.49		Saponin concentration in short method, 480 mg. per 100 cc.
5	10.23	10.45	0.24	Saponin concentration in short method, 70 mg. per 100 cc.
		10.49		Saponin concentration in short method, 480 mg. per 100 cc.
Average of differences.....			0.12	

In order to get the greatest accuracy, the figure for the old method in each case represents an experiment where there was no solution of the stromata. Duplicates of 3, 4, and 5, may be found opposite 3, 5, and 6, respectively, in the table in Section 5 of Part C.

The close checking of the duplicates in the short method in spite of the widely varying amounts of saponin is noteworthy.

The short method evidently gives slightly higher results than the long method, the average being 0.12 per cent. The average of the last two experiments is believed to represent a more exact

figure, since they were done last, with the greatest care. This means a difference of 0.25 per cent of hemoglobin. It is an interesting question why the short method does not give higher values on account of the complete solution of the stromata which takes place. It was found (Section 5 of Part C) that the stromata added to the refractive index an amount equivalent to 0.45 per cent in terms of hemoglobin concentration. The unaccounted for difference is $0.45 - 0.25 = 0.20$ per cent. As a possible explanation is advanced the theory that when the red cells dissolve there is no longer the inequality in the distribution of inorganic constituents between cells and plasma that there was before. The refractive index of the inorganic constituents of red cells and plasma was calculated from Schmidt's tables, and found to be (in terms of excess refraction over that of water at the same temperature):

Plasma.....	107.2
Red cells.....	73.30
	<hr/>
Difference	23.9

The uncertainty of the analyses makes an estimation of the differences in non-protein organic constituents unsatisfactory.

This difference in terms of hemoglobin percentage would be

$\frac{23.9}{194.2} = 0.12$ per cent and would tend to make the hemoglobin estimated by the short method low by that amount. There remains then only $0.2 - 0.12 = 0.08$ per cent to be accounted for, and this may easily be the experimental error.

In conclusion it may be stated that the close checks in the methods tabulated above are attainable only by the greatest care in all operations.

CONCLUSIONS.

The short method gives even closer checks than the long method. The results by it are 0.25 per cent of hemoglobin too high. It is advised that it be used with this correction, in preference to the long method, because there is no variation in the degree of solution of the stromata.

SUMMARY.

The refractive constant "*a*" for human hemoglobin, determined on pure hemoglobin solutions, the concentration of which was obtained by getting the dry weight at 110°C., was found to be 194.2. This compares with 183 found by Howard on crystalline horse hemoglobin, and, if the substances are comparable, would indicate 5.7 per cent of water of crystallization.

The subtraction of the refractive index of water at the temperature of the hemoglobin solution from the refractive index of the hemoglobin solution gives a figure proportional to the hemoglobin concentration over a very wide range.

Hemoglobin in the presence of NaCl and at a pH close to the isoelectric point of 6.8 can be coagulated in 3 minutes at 100°C., giving a clear filtrate.

The refractive indices of the filtrates from the heat coagulation of hemoglobin correspond very closely to the ultrafiltrates from the same solutions. This gives an easy way to separate the refractive effect of hemoglobin from that of non-protein constituents under the proper conditions.

A method is described for obtaining the concentration of hemoglobin in terms of dry weight of the substance, applicable to blood.

This method compared with the oxygen capacity of the same blood obtained by the Van Slyke method, gives a ratio of 1.26, as compared with Hüfner's of 1.34. If the oxygen capacity were obtained by the Haldane method the ratio would be about 1.15.

A short method of obtaining the hemoglobin concentration by the refractometer is described. This method checks closely with the longer and more conclusive method.

We wish to express our indebtedness to Dr. Arlie V. Bock and Dr. Henry Field, Jr., for the determinations of the oxygen capacity.

LEAD STUDIES.

VIII. THE MICROCHEMICAL DETECTION OF LEAD.

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From the view-point of the clinical laboratory, the accurate determination of lead in biological material is a relatively complicated procedure. The several factors which account for this have already been discussed (1). A relatively simple qualitative test for lead would therefore accomplish two things: (a) It could be used to confirm the quantitative determination of the small amounts used; and (b) it could be readily used in the clinic where only qualitative results are usually desired. It was for these reasons that distinctive microchemical methods were studied.

The so called "triple nitrite test" of Behrens and Kley (2) is perhaps the most characteristic of the microchemical tests for lead. It depends upon the formation of a hexanitrite of potassium, copper, and lead— $\text{K}_2\text{CuPb}(\text{NO}_2)_6$. This hexanitrite is readily identified under the microscope by its regular crystalline structure. For this reason the test is extremely delicate and far surpasses the usual microanalytical tests for lead, such as the iodide and chromate tests. The iodide test is not particularly distinctive under the microscope, nor is it wholly satisfactory because of the relative solubility of lead iodide. Lead chromate, while very insoluble, is invariably precipitated in an amorphous condition so that identification is difficult.

The triple nitrite test consists in adding small crystals, successively, of copper acetate, sodium acetate, and potassium nitrite to a drop of the lead solution which had been slightly acidified with acetic acid. Sodium acetate is added in order to buffer free mineral acids, which interfere with the test. The hexanitrite crystallizes in regular rectangular plates or cubes (Figs. 1 and 2),

usually black, but occasionally dark red when the plates are thin. With relatively large amounts of pure lead salt the test seldom fails when made in this way. Frequent failures have been obtained, however, when this test was applied to traces of lead precipitated in the analysis of biological material. Here it frequently happens that the lead is present in such small amounts that considerable analytical skill is necessary to make an exact estimation. A study was therefore made for the purpose of applying this test to clinical material and for defining more exactly the conditions under which the hexanitrite would crystallize in low concentrations.



FIG. 1.

FIG. 2.

FIG. 1. Showing the regular rectangular plates or cubes of hexanitrite crystals. $\times 85$.

FIG. 2. Showing the regular rectangular plates or cubes of hexanitrite crystals. $\times 350$.

In order to insure constant results with this test it was found that definite quantities of reagent must be used; the procedure as usually outlined is altogether too indefinite. Instead of adding crystals of the solid salts, therefore, the reagents in solution form should be measured from capillary pipettes. The latter may be made readily by drawing out a piece of 4 mm. glass tubing to a capillary and blowing a bulb at one end. The stem may be calibrated roughly by marking two points, representing a volume of 5 c. mm. (about $\frac{1}{10}$ of a normal drop). Using measured quanti-

ties of reagents of definite concentration results not only in reproducible results but also avoids any tendency to "drown" the preparation—a frequent cause of failure noted by Schoorl (3). Solutions of the following composition are required: Sodium acetate 4 per cent, pure copper acetate 2 per cent, and 10 per cent acetic acid.

The purity of the lead solution to be tested is a decided factor in the success of the test. The lead must be separated in such a way therefore that contamination is reduced to a minimum. Ammonium, iron, and calcium salts interfere with the test, even when a relatively large amount of lead is present. The most convenient method of separating the lead is as the sulfide. This is possible even with a very minute amount of lead in the presence of a relatively great concentration of other inorganic salts. The means of accomplishing this, however, must be a refinement of the ordinary analytical procedure. The acidity of the solution must be reduced as far as possible in order to precipitate the maximum amount of lead; for this reason the solution should be made just acid to methyl orange by the least amount of hydrochloric acid possible. The physical separation of the colloidal lead sulfide presents another difficulty. 0.005 mg. of lead in 15 to 20 cc. of solution gives a barely visible yellow color on saturation with hydrogen sulfide. Centrifugation alone is insufficient to throw out the lead sulfide in such a case. A convenient means of accomplishing this, however, is to add first of all a drop of the 2 per cent copper acetate solution and 1 cc. of a saturated solution of ammonium sulfate. The ammonium sulfate serves to coagulate the colloidal sulfides and the copper sulfide on centrifugation carries down the lead sulfide by *entrainment*.

Since traces of inorganic salts interfere seriously with the test, these must be removed by careful washing. This is readily accomplished by decantation, as the sulfide packs well in the bottom of the centrifuge tube. If the washing is carefully done it is usually unnecessary to reprecipitate the lead as sulfide.

Cesium or thallium salts improve the sensitiveness of the test for lead, as the corresponding hexanitrites are much more insoluble than the potassium hexanitrite. With thallium the sensitiveness is particularly increased. However, the crystals of the thallium compound are very minute and much more difficult to identify than

the potassium salt. Also crystals of thallous chloride, which occasionally form, are apt to cause confusion. For the clinical detection of lead, the more common potassium nitrite is quite sufficiently sensitive as a reagent.

Procedure.

In testing for lead in organic material, it is essential to oxidize the organic substances present before lead will respond to the ordinary precipitation reactions. The material should be baked and ashed and the ash completely dissolved in hydrochloric acid.¹ The volume of solution should be about 25 cc. After neutralizing the solution thus secured it should be made just acid to methyl orange with a small amount of dilute hydrochloric acid. 1 cc. of a saturated solution of ammonium sulfate and 1 drop of 2 per cent copper acetate solution are added and the cold solution is saturated with hydrogen sulfide gas. The sulfides are thrown out by centrifugation and washed at least three times by decantation, the water being drained completely from the precipitate each time by means of a capillary tube. The washing must be thorough and the wash water completely drained from the sulfide precipitate each time, as small amounts of inorganic salts seriously interfere with the test. The centrifuge tube containing the sulfides, drained free from water, is then placed in a beaker of boiling water and 2 drops of nitric acid are added. Some of the solution thus obtained should be drawn up into a capillary tube and a drop or so evaporated to dryness on a microscope slide. 5 to 10 c.mm. of 4 per cent sodium acetate solution should then be added and the residue completely dissolved. In case copper acetate was not added previously to aid in the precipitation of the lead sulfide, it is necessary also to add 5 c.mm. of the 2 per cent copper acetate solution. The residue must be thoroughly mixed with these so that all particles dissolve. The material should be collected into 1 drop-let and again evaporated to dryness. When carefully done the residue will consist of a rim about 4 mm. in diameter in which the greater part of the salts are concentrated. The slide should then be chilled on ice, 5 c.mm. of 10 per cent acetic acid and a small crystal of potassium nitrite are added. This nitrite solution should

¹ Fairhall (1), p. 14.

be centrally placed in the dry residue and allowed to diffuse to the outer edge. This slow diffusion permits the hexanitrite crystals to form at various places under optimum conditions of concentration. The small black cubes and squares are usually found in the outer rim scattered among the crystals of copper acetate. Instead of adding a small crystal or two of potassium nitrite to the acetic acid droplet, 5 c.mm. of a 20 per cent potassium nitrite solution acidified with acetic acid may be added directly. The former method is the more convenient, however, since the acid-nitrite solution rapidly decomposes. An excess of potassium nitrite serves to reduce the solubility of the hexanitrite, though this is opposed to the statement of Chamot (4) that the hexanitrite is soluble in an excess of the salt. That this is not so may be shown by adding solid potassium nitrite to a saturated solution of the hexanitrite. This produces an immediate precipitate of the black crystals showing that an excess diminishes the solubility instead of increasing it. Therefore, a slight excess of potassium nitrite is an advantage. However, an excess of acetic acid solution is to be avoided, as the crystals are rather soluble in water. The amount of acetic acid is restricted for this reason to 5 c.mm.

By evaporating a drop of a very dilute solution of a pure lead salt on a microscope slide it is possible to estimate the sensitiveness of limit of this test for lead. This sensitiveness is given as $0.03\mu\text{gm.}$ by Behrens and Kley² and $0.003\mu\text{gm.}$ by Emich (5). It is quite out of the question, however, to expect to separate any such small amount of lead from several grams of ash and to identify it. Indeed, 2 or $3\mu\text{ gm.}$ of lead salt dissolved in 15 to 20 cc. of water fail to show the slightest color change with hydrogen sulfide. With the above procedure, however, experiments repeatedly have shown that 0.001 mg. or $1\mu\text{ gm.}$ of lead may be separated and readily identified from 15 cc. of a solution containing other salts.

Effect of Bismuth.

A great many of the reactions of bismuth are so similar to those of lead that it is somewhat difficult to identify either metal when present in amounts less than 1 mg. Since bismuth salts are occasionally administered medicinally in cases of lead poisoning,

² Behrens and Kley (2), p. 65.

the distinction between the two must be closely drawn. When bismuth is present a white precipitate is produced by sodium acetate. This precipitate obscures or prevents the formation of the hexanitrite. The greater part of the bismuth may be separated, however, by converting it into the insoluble basic nitrate by dilution with water. The most satisfactory method is to evaporate the nitric acid solution of nitrates in the centrifuge tube to dryness and then add 1 or 2 drops of water. A white precipitate of the bismuth oxynitrate forms, while the lead nitrate passes into solution. A drop of this solution may be filtered readily in a modified Savage filtration tube (6). For this purpose a fine capil-



FIG. 3.

FIG. 3. Showing the hexanitrite crystals formed in the presence of a small amount of bismuth. $\times 350$.

lary tube having a tiny plug of absorbent cotton at one end is dropped into the solution. The clear liquid is drawn into the tube by capillary action and, after breaking off that part of the tube containing the plug, may be transferred to the slide. The small amount of bismuth left in solution no longer interferes with the test for lead. Fig. 3 is a photomicrograph of such a test where the ratio of bismuth to lead was originally 100:1. The very slight amount of bismuth still present separates as an amorphous precipitate, but does not prevent the formation of the hexanitrite crystals.

Purification of Reagents.

Most of the reagents employed for the separation and identification of lead will be found to contain that metal in minute amounts. Purification of these reagents is therefore a necessary first step in the application of this test for lead. Fortunately this is a simple matter in most cases. With ammonium sulfate, for example, the cold solution may be saturated with hydrogen sulfide gas, allowed to stand over night, the solution filtered from the slight precipitate of sulfide, and the excess hydrogen sulfide removed by boiling. Potassium nitrite often contains a relatively large amount of lead. A very pure grade of potassium nitrite may be prepared from silver nitrite. In order to do this, silver nitrate should be added to the filtered solution of potassium nitrite and the resulting precipitate of silver nitrite washed well with cold water. This silver nitrite is then dissolved in boiling water and an equivalent amount of pure potassium chloride added. The pure potassium nitrite in solution may then be crystallized by evaporating this solution to a small bulk. Acids and ammonia may be purified by the method devised by Lenz (7).

CONCLUSION.

A modified procedure for the hexanitrite test for lead has been described and applied to the detection of lead in biological material. By means of this method as small a quantity as 1μ gm. of lead may be separated and identified in the presence of a considerable amount of other inorganic salts. The test may be used as a satisfactory and relatively simple clinical method for the detection of lead.

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COMPARATIVE STUDY OF THE CONCENTRATION OF VARIOUS ANIONS AND CATIONS IN CEREBRO- SPINAL FLUID AND SERUM.

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Cerebrospinal fluid has been analyzed chemically by a number of investigators (1). These have confined themselves usually to the quantitative estimation of well known organic constituents or of the inorganic constituents as a whole. Mestrezat (2) determined the concentration of the individual inorganic elements in cerebrospinal fluids, using for the purpose standard gravimetric and volumetric methods. For these methods large amounts of cerebrospinal fluid were required. It was therefore necessary to pool the fluid from several individuals. Recent improvements in analytical technique have made possible the accurate quantitative estimation of the concentration of all the well known elements (sodium, potassium, calcium, chlorine, phosphorus, and bicarbonate), in such a volume of cerebrospinal fluid as may readily be obtained from a single patient. Depisch and Richter-Quittner (3) in a very elaborate study determined the concentration of a number of substances in the cerebrospinal fluid of normal individuals as well as in that of individuals suffering from various diseases. They also simultaneously determined the concentration of some of the substances in the serum of the same patients. The variation in the concentration of certain of the inorganic elements as found by these workers is so great as to justify serious scepticism as to the accuracy of their determinations. It is now well known from studies on serum that the concentration of the inorganic constituents of serum is remarkably constant during health and varies but little in disease. Where

definite variations are found they are usually associated with definite clinical symptoms. It has been conclusively shown that the calcium concentration of the serum of the normal adult is constant, varying only within the narrow limits of 9.5 and 10.5 mg. per 100 cc. of serum. In no condition is the calcium concentration of serum increased above the normal level. Depisch and Richter-Quittner report 14.84 mg. per 100 cc. as the calcium concentration of the serum of a normal adult. For pathological sera values ranging from 19.6 to 53.53 mg. per 100 cc. of serum are given. It is quite impossible to explain such results.

The work which furnishes the basis of this report was undertaken to determine:

1. The concentration of the cations (sodium, potassium, and calcium) and the anions (chlorine, phosphate, and bicarbonate) in normal cerebrospinal fluid.

2. The changes in the concentration of these elements with disease.

3. By analyzing the serum and cerebrospinal fluid obtained simultaneously from the same patient we attempted to determine the nature of the equilibrium that prevails between these two body fluids, the former with its high protein content, the latter protein-free.

We have studied the cerebrospinal fluid and serum of thirteen patients. Three of these patients were normal. The others were suffering either from some general constitutional disease or from some specific involvement of the central nervous system.

Method.

Lumbar and venous punctures were performed in the usual manner. Four of our adult patients were in the sitting position, the remainder were lying down during the procedure.

Cerebrospinal fluids and sera were examined at once. Only fluids free of blood were examined. Hemolyzed sera were rejected.

Sodium, calcium, and potassium were determined by the methods devised by Kramer and Tisdall (4). Phosphorus determinations were made by employing the Brigg's modification (5) of the Bell-Doisy method. We used Whitehorn's method (6) in our chlorine and Van Slyke and Cullen's method (7) for our CO_2 determinations.

TABLE I.*
Concentration of the Inorganic Constituents of Blood Serum and Spinal Fluid of Normal Individuals.

Patient.	Sex.	Age.	Diagnosis.	Ca		P		Cl (NaCl)		K		Na		CO ₂	
				Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.
C. B.	Male.	60	Normal.	10.5	5.0	3.2	1.8	570	720	20.0	12.8	340	370	59	62
A. T.	"	28	"	10.5	5.0	3.2	1.1	613	740	21.0	16.0	336	336	60.5	57
A. B.	"	20	"	10.6	4.4	2.3	0.8	574	735	24.2	16.6				

* The results, except those for CO₂, are expressed in mg. per 100 cc. of fluid.

TABLE II.*
Concentration of the Inorganic Constituents of Blood Serum and Spinal Fluid in Individuals Suffering from Various Diseases.

Patient	Sex	Age, yrs.	Diagnosis	Ca		P		Cl, NaCl		K		Na		CO ₂	
				mmol/l.	mg. spinal fluid	mmol/l.	mg. spinal fluid	mmol/l.	mg. spinal fluid	mmol/l.	mg. spinal fluid	mmol/l.	mg. spinal fluid	mmol/l.	mg. spinal fluid
R. S.	Male.	8	Acute nephritis.	8.8	5.4	6.16	1.7	614	674			300	298	50	47
A. B.	Female.	36	Cerebrospinal lues.	9.9	5.3	4.0	1.0	561	709	17.4		296	310	57	55
A. J.	Male.	33	"	10.5	4.6	2.8	1.4	594	726			298	305		
C. W.	"	15 mos.	Tuberculous meningitis.	8.2	4.9	2.5	1.1	594	701	19.2	12.8				
L. D.	"	40	Cerebrospinal lues.	10.2	4.6	3.1	1.0	570	709	20.5	15.8	350	370	58.6	60.7
W. B.	"	37	Paresis.	9.4	4.6	3.6	1.1	566	705	23.6	16.3	342	363	57.0	58.0
S. H.	"	65	"	10.0	5.0	2.8	1.9			23.0	17.0	362	370	47.0	50.0
J. B.	"	36	Cerebrospinal lues.	10.0	5.0	3.0	1.8	597	742	19.8	13.6	354	360		
N. R.	"	10	Paresis ?	10.0	5.0	2.2	1.5	612	725	19.1	12.0	327	359		
W.	"	13	Epilepsy.	10.0	5.1	4.1	1.3	550	752	19.0	12.9	337	345		

* The results, except those for CO₂, are expressed in mg. per 100 cc. of fluid.

Table I gives the results of determinations made upon the serum and spinal fluid of three normal individuals. The calcium concentration of the spinal fluid is found to be 40 to 50 per cent of that of the serum of the same individual or 4.4 to 5.0 mg. per 100 cc. of spinal fluid. These figures agree very well with those obtained by Neuhausen and Pincus for the free calcium of serum. The inorganic phosphorus concentration of spinal fluid is much less than that of the serum of the same individual. The chlorine concentration, however, is much higher in cerebrospinal fluid than in serum. This is a regular finding not only with normal but also with pathological fluids. The potassium concentration is always less in spinal fluid than in the serum, varying from 12.8 to 16.6 mg. per 100 cc. of cerebrospinal fluid as compared with 20.0 to 24.2 mg. of the same element in serum. We are unable to explain the high results obtained by Depisch and Richter-Quittner who found as much as 72 mg. of potassium per 100 cc. of normal serum. In an unpublished report Kramer and Wilkins have shown that the potassium concentration of normal serum varies only from 18 to 24 mg. per 100 cc. The sodium concentration of spinal fluid is essentially the same as that of serum. The same applies to the bicarbonate concentration.

Table II contains the results of analyses of the blood serum and spinal fluid of ten patients suffering from various diseases. The calcium concentration of the spinal fluid remains remarkably constant even though that of the serum may at times be somewhat decreased. The inorganic phosphorus of the spinal fluid is regularly very low, quite independently of the concentration of the same element in the serum. As with normal individuals so we find here that the chlorine concentration of spinal fluid is much higher than that of the serum of the same patient. The relationship between the potassium, sodium, and bicarbonate of spinal fluid and serum is the same as in the normal individual.

In Table III the results for normal fluids are summarized.

DISCUSSION.

We have no desire to enter here into a discussion as to the nature of cerebrospinal fluid or the mechanism of its formation. These subjects have been reviewed at length by Dandy and Blackfan (8), by Weed and Cushing (9), and more recently by Becht (10).

Cerebrospinal fluid is considered by some to be a secretion, whereas others look upon it as being an ultrafiltrate or a dialysate. Normal cerebrospinal fluid contains the same electrolytes as serum, but practically no protein whereas serum contains 7 to 9 per cent of protein. The proteins of serum do not diffuse through a collodion membrane. Donnan (11) has shown that when a membrane

TABLE III.

Average Concentration of the Anions and Cations in Normal Serum and Spinal Fluid.

Average values expressed in mg. per 100 cc. of material.

Material.	Ca	P	Cl (NaCl)	K	Na	CO ₂
Serum.....	9.6	2.9 (Adults.) 4.6 (Children.)	578	20.9	328	55.6*
Cerebrospinal fluid.....	4.8	1.3	712	14.7	351	55.7

* Concentration expressed in cc. of CO₂ gas per 100 cc. of serum at 0°C. and 760 mm. of Hg.

TABLE IV.

Concentrations of Anions and Cations in Cerebrospinal Fluid and Serum Expressed in Gram Molecules per Liter.

Material.	Sodium.	Potassium.	Calcium.	Chlorine.	Bicarbonate.	Phosphate.
Serum.....	0.143	0.005	0.0025	0.102	0.032	0.001
Cerebrospinal fluid.....	0.143	0.004	0.0012	0.124	0.032	0.0006
				Serum.*	Cerebrospinal fluid.	
Total concentration of cations.....				0.150	0.148	
“ “ “ anions.....				0.135	0.157	
Product.....				0.020	0.023	

* *Note.*—If we assume that only 92 per cent of the serum is water and calculate the concentrations accordingly the agreement is much better.

$$\begin{array}{rcl}
 \text{Concentration of cations in serum} & = & 0.163 \text{ molal} \\
 \text{“ “ anions “ “} & = & 0.147 \text{ “} \\
 \text{Product “ “} & = & 0.024 \text{ “}
 \end{array}$$

separates two solutions of electrolytes, one of which contains one ion which cannot diffuse through the membrane while all the other ions can diffuse through the membrane, the result will be an unequal distribution of the diffusible ions on the opposite

sides of the membrane. At equilibrium the products of the concentration of each pair of oppositely charged diffusible ions are the same on the opposite sides of the membrane.

In Table IV are given the molal¹ concentrations of the various anions and cations in serum and cerebrospinal fluid. When the figures for the concentrations of the oppositely charged ions in each fluid are multiplied by each other the results are approximately the same both for serum and cerebrospinal fluid. We are aware of the fact that no account has been taken of differences in ionization of different salts, but sodium and chlorine which constitute the major part of the electrolytes are both practically completely ionized in blood serum and very likely also in cerebrospinal fluid. In a study of the physical and chemical properties of serum, and serous effusions Loeb, Atchley, and Palmer (12) found the potassium content of serum to be always higher than that of the effusion. The chlorine content of the serous fluid is always higher than that of the serum, while the sodium and bicarbonate are equally distributed. When the serum and the effusion were dialyzed against each other the equilibrium between the electrolytes remained unaffected.

Table I shows that a similar equilibrium exists between serum and cerebrospinal fluid. Here also the chlorine concentration of the spinal fluid (protein-free) is higher than that of the serum, the sodium and bicarbonate concentrations are the same, whereas the potassium concentration is lower in the spinal fluid than in the serum. Some determinations of the calcium of serum and serous effusions have shown the calcium concentration to be about the same in both fluids, whereas here we find the calcium concentration of spinal fluid to be only about 50 per cent of that of the serum. However, serous effusions always contain a variable amount of protein, whereas normal spinal fluid is practically protein-free.

CONCLUSIONS.

The concentration of the various cations and anions in spinal fluid and serum of the same patient has been determined.

A study of the equilibrium between the concentration of these elements in the serum and spinal fluid suggests that the Donnan membrane equilibrium plays an important part.

¹Since practically all the ions are univalent, the gram equivalent and molal concentrations are the same.

We take pleasure in expressing our thanks to Dr. Franklin of Bay View Hospital for his assistance in securing much of the material for study.

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ASTERIASTEROL—A NEW STEROL FROM THE STARFISH AND THE STEROLS OF CERTAIN OTHER MARINE ECHINODERMS.

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The present study was undertaken with the view to obtaining information of a chemical nature that might help to explain some of the curious differences that exist between the eggs of the starfish (*Asterias forbesi*) and other echinoderms; more especially those of the sea urchin (*Arbacia punctulata*) and sand dollar (*Echinarachnius parma*).

Mathews (1) some time ago investigated the sterols of the starfish (*Asterias forbesi*) and the sea urchin (*Arbacia punctulata*), but was unable to demonstrate the presence of true cholesterol in the starfish egg, but did find a substance in the sea urchin that exhibited the properties of cholesterol.

Kossel and Edlbacher (2), in analyzing a different species of starfish (*Astropecten auranticus*) found rather large quantities of a cholesterol isomer which he termed stellasterol. Dorée (3) found a peculiar sterol or probably mixture of sterols from *Asterias rubens*, but according to Kossel, from solubility studies, not identical with his stellasterol. Dorée believes one of the substances that he isolated to be a complex alcohol or hydrocarbon, while the other shows points of resemblance to the members of the sterol group.

EXPERIMENTAL.

The following mode of analysis was found most suitable for the separation and identification of the sterols of both the bodies and the eggs of the echinoderms studied.

Method.

In the case of the eggs, the ovaries were carefully separated by straining through cheese-cloth. The eggs were then centri-

fuged in order to concentrate, and dried carefully on a water bath. The bodies were cut up while alive, dried on a water bath, and ground to a fine powder with washed sand.

Extraction was carried out with anhydrous ether three successive times, each extraction lasting 5 hours. This process was followed by a 95 per cent ethyl alcohol extraction lasting 8 hours, the extracts were combined, and the lecithin fraction was precipitated with an excess of acetone. The ether and acetone were then evaporated off at room temperature and the residue

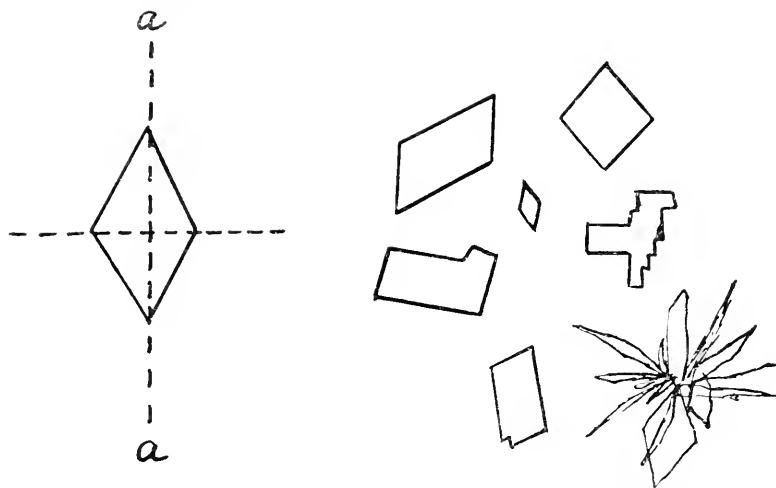


FIG. 1.

FIG. 1. Asteriasterol recrystallized from alcohol. Extinction, oblique; birefringence, low; least elasticity, aa; elongation, +.

was immediately taken up in hot 95 per cent alcohol. To this solution another hot alcoholic solution containing more than sufficient digitonin to precipitate the sterol was added, and the mixture heated on a water bath for 10 minutes. This mixture was then placed in an ice box for 12 hours to insure complete separation of the sterol-digitonide. The precipitate was filtered off, carefully dried at 60°C., boiled with an excess of acetic anhydride for 10 minutes, and the solution poured into a large excess of cold water.

The resulting precipitate of the sterol acetate was filtered off, saponified with alcoholic potassium hydroxide, poured into an

excess of cold water, filtered off, washed with water, and dried. The mass was then recrystallized once from ethyl acetate and three times from ethyl alcohol. (Fig. 1.)

An alternative method consisted of extracting with ether and alcohol, saponifying the combined extract with sodium ethylate, filtering off the insoluble soaps, converting the unsaponifiable material to the acetate, saponifying with NaOH, and finally recrystallizing from absolute ethyl alcohol. (Fig. 2.)

Preparation of the benzoate part of the residue from the ether alcohol extraction was taken up in pure pyridine, an excess of benzoyl chloride added, and the mixture allowed to stand over-

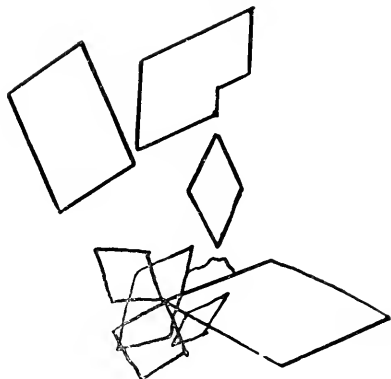


FIG. 2.

FIG. 2. Asteriasterol acetate crystallized from absolute alcohol.

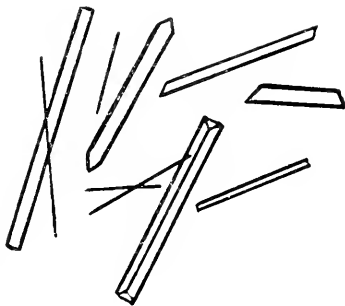


FIG. 3.

FIG. 3. Asteriasterol benzoate recrystallized from absolute alcohol.

night. It was then poured into an excess of cold water, filtered off, washed thoroughly dry, and recrystallized four times from 95 per cent ethyl alcohol. (Fig. 3.) An orange oil was very often obtained on adding the pyridine system to water. This was found to crystallize if cooled for a few hours, but if this failed, gentle warming on the water bath and subsequent cooling produced the desired result.

It was found that with the residues from the extraction of almost exhausted portions of eggs that on solution with pyridine a stiff jelly was obtained. The substances involved were not further investigated, but it may be of interest to note that Dorée (3) had

a similar experience with the benzoations of the sterols of *Asterias rubens*. This jelly gives a decidedly positive reaction with the Schiff test for cholesterol in the case of *Arbacia*.

Results of Analysis.

Properties of the sterol (asteriasterol) from *Asterias forbesi* are as follows:

1. With the Liebermann-Burchard reaction an intense purple-blue flashed out immediately on the addition of the sulfuric acid, and within 5 minutes the color had almost entirely disappeared. This test was always performed using 10 cc. of chloroform solution of the sterol plus 2 cc. of acetic anhydride and 1 cc. of concentrated H_2SO_4 ; the tube tapped for mixing and placed in the dark until the color had reached a maximum. With true cholesterol the color develops slowly and reaches its height within approximately 15 minutes, slowly fading out to a yellowish green.

2. This sterol was easily soluble in ether, alcohol, acetone, chloroform, and other lipid solvents.

3. Salkowski's reaction was atypical.

4. Without previous oxidation by means of benzoyl peroxide a reaction very similar to that obtained with oxysterol using the Lifschütz reaction (4) was found.

5. Rosenheim's (5) monomethyl sulfate test was indecisive.

6. Golodetz' modification (6) of Tschugajeff's test was negative.

7. Lifschütz' reaction (4) gives a slightly purplish brown coloration whereas cholesterol itself gives a brilliant emerald green.

8. This substance gives no precipitate on heating with *Quillaja saponaria*.

9. It forms a digitonide, but probably not quantitatively.

10. Asteriasterol reacts with Whitby's reaction A reagent (7) to give a yellowish red with greenish fluorescence. The purple in the $CHCl_3$ layer was not present. Acetic anhydride gave a reddish yellow color to this layer but no blue, characteristic of cholesterol.

11. Whitby's (7) reaction B gave a distinct reddish yellow, but no purple. This reaction with true cholesterol is most striking.

12. With cold $AsCl_3$ (Kahlenberg, 8) a yellow-brown was produced which color on heating turned reddish brown. True cholesterol gives a remarkably beautiful cherry-red.

13. The melting point of asteriasterol was $\pm 70^\circ C.$; that of the acetate was $97^\circ C.$; and that of the benzoate was $125^\circ C.$

The Whitby reactions are very striking indeed and surely represent a distinct advance in the means whereby sterol isomers may be differentiated. The $AsCl_3$ reactions of Kahlenberg are also of great importance.

Sterols of Other Organisms.

Sand Dollar (Echinarachnius parma).—The sterol from this organism is undoubtedly true cholesterol. The material gave a good rose-red with dimethyl sulfate which color was readily discharged with alcohol. Heated with benzoyl peroxide and acetic anhydride to dryness, taken up in chloroform, and boiled with dimethyl sulfate it gave a deep raspberry-red. When ferric chloride and acetic anhydride were added it gave a deep green coloration with a precipitate. These reactions are characteristic of true cholesterol.

With the Lifschütz reaction a brilliant emerald green, characteristic of cholesterol was obtained. The Liebermann-Burchard reaction gave a coloration identical with that obtained with true cholesterol. Whitby's reactions A and B were both characteristic of true cholesterol.

Sea Urchin (Arbacia punctulata).—Crystals identical with those of true cholesterol have been isolated. The Liebermann-Burchard reaction and Whitby's reactions A and B are characteristic of true cholesterol. With AsCl_3 at room temperature a distinct purple was produced, changing to brownish purple on standing. Heating produced a greenish brown changing to yellow-brown, similar to hydrous lanolin. This would indicate the presence also of ischolesterol.

Cumingia tellinoides.—The Liebermann-Burchard and Schiff reactions were characteristic for true cholesterol.

Choctopterus pergamentaceus.—The Liebermann-Burchard and Schiff reactions were characteristic for true cholesterol.

Arenicola cristata.—The eggs plus their jelly of *Arenicola cristata* gave a sterol that exhibited the Liebermann-Burchard reaction as found with true cholesterol.

 AsCl_3 Reaction for Oxysterol.

Oxysterol prepared by oxidation of cholesterol with benzoyl peroxide was employed. The material dissolved in AsCl_3 immediately produces a beautiful deep violet which on gently heating turned bright blue and on vigorous boiling, bright green. The color changes were remarkably sharp and brilliant.

Little has been done toward the isolation and identification of the sterols of various organisms, but what meager data there are available indicate a very large number of isomers of cholesterol. Windaus and von Staden's recent work (9) has shown chemically how very differently some of these isomers may react. Their biological significance is undoubtedly very important.

SUMMARY.

1. The sterol of the starfish (*Asterias forbesi*) has been isolated, identified, and named.

2. The sterols of the sand dollar (*Echinarachnius parma*) and the sea urchin (*Arbacia punctulata*) have been found to be identical with true cholesterol.

3. The sterols of *Cummingia tellinoides*, *Choctopterus pergamentaceus*, and *Arenicola cristata* very probably are identical with true cholesterol.

4. The use of AsCl_3 as a means of identifying sterols has been extended to oxysterol. The reaction is very striking.

I wish to express my sincere appreciation to Dr. G. H. A. Clowes for his unvarying interest and enthusiasm during this investigation.

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THE ACCURACY OF THE "IONOMETRIC" METHOD AND OF THE PROTEIN CORRECTION IN MEASURING SERUM CONDUCTIVITY.

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(Received for publication, June 1, 1923.)

The conductivity method for studying the electrolyte concentration in the blood serum has not been widely used in clinical studies. This has been due largely to difficulties inherent in the rather complicated apparatus which has been necessary and to the large amount of serum that has been required.

Previous reports (Gram (1), Gram and Norgaard (2)) have demonstrated that relatively large variations in the conductivity of serum occur in various clinical conditions and that these variations may be detected by the use of the ionometric method of Johanne Christiansen (3). This method is so simple and so economical of material that it would seem to afford the method of choice for clinical studies provided it is sufficiently accurate.

To determine the degree of accuracy which can be obtained with it, we have compared the method with the standard Kohlrausch method using both pure salt solutions and serum. It will be shown that the method is accurate to within about 1 per cent and that it is admirably suited to clinical and physiological investigations.

Method.

Kohlrausch Method.—The Kohlrausch bridge used was of the Leeds and Northrup type. The alternating current was supplied by an audiooscillator; with the adjustable head phones and the

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Ostwald type of cell used, the sensitivity was about 0.0005 of the bridge length. All the determinations were made at 20° ($\pm 0.05^\circ$) with a manually controlled water bath.

The Ionometer of Johanne Christiansen.—Since the original description of the apparatus is not generally available the apparatus is described in some detail.

The method utilizes the principle that for resistances in series the E.M.F. across each resistance is proportional to that resistance. If a voltmeter of known resistance is used with a current of constant voltage and if an unknown resistance R_x is then inserted in series with the voltmeter the value of the unknown resistance may be calculated from the two voltmeter readings: $R_x = \frac{S_e - S_x}{S_x} R_e$ where R_e and R_x are the voltmeter and unknown resistances and S_e and S_x are the readings of the voltmeter without and with the unknown resistance in series. The apparatus used consists of a spring voltmeter for either D. C. or A. C. arranged with two contact buttons to connect the voltmeter either directly across the house current or with the conductivity cell containing the unknown solution in series with the voltmeter. This arrangement allows rapid comparison of the two voltmeter readings. To simplify the calculation the voltmeter is so arranged as to be adjustable to a standard reading when measuring the house current alone. In the instrument used in this work this adjustment is taken care of by a "magnetic shunt." Accuracy of reading is increased by a mirror background for the pointer. The scale is divided into 120 divisions and readings can be made to one-fourth of one division.

The conductivity cell consists of a U-shaped capillary tube, the capillary expanded at each end into cups, fitted with bright platinum wires sealed in and pressed against the glass in such a manner that there is little danger of disturbing their position. The minimum amount of fluid required is about 0.5 cc. The vessel is mounted in an ebonite holder with contact plugs to which the platinum wires are soldered and the whole is connected with the voltmeter in the same manner that one plugs in an ordinary electric connection.

The temperature is controlled to within 0.1°C. by means of a tumbler of water which is so placed that the cell is immersed to above the electrode level.

The advantages of this type of conductivity apparatus are the readily available current, the simplicity and portability of the apparatus, the unchanging electrodes which require a minimum of care, the rapidity of determination, and the economy in material to be tested.

The error due to polarization and temperature change is less, with this apparatus, than is the error involved in the reading.

The necessity for the use of alternating current to avoid polarization is avoided by the high resistance of the conductivity cell. Either D. C. or A. C. current may be used with an appropriate voltmeter.

Routine determinations are made on duplicate samples taking four to six successive readings on each sample, the reading with the house current being taken between each reading of the unknown. If change in voltage of the house current occurs the readings are repeated after adjustment of the voltmeter to the standard initial reading. When serum is used a little clot of protein will form around the anode. This should be removed with a soft brush before the next examination.

Standardization of the Ionometer.—The scale values of the voltmeter must be converted to some units of conductance or resistance. The sodium chloride equivalents of conductivity, that is the percentage of NaCl in water giving the same conductivity as the solution under examination, have been adopted in previous work. The temperature of 20°C. was chosen as that nearest the average temperature of the laboratory. The solutions for standardization were carefully made up by preparing a solution of 10 gm. of dry pure NaCl in distilled water to 1,000 cc. From this standard solution the different solutions were made up by adding varying amounts from a calibrated burette into a calibrated 50 cc. measuring flask and making up to the mark with redistilled water. For further control the salt solutions were titrated for chlorides according to the Austin-Van Slyke method and found correct. The results of such a standardization for the range of serum are presented in Table I, Columns 1 and 2, and as a curve in Fig. 1. The figures are given simply as an example as it is necessary to standardize each apparatus. In practise the portion of the curve between 0.500 and 0.800 per cent NaCl equivalents is plotted on a large scale against the scale reading of the voltmeter. For urine it is necessary to extend the range of the standardization. It is for many purposes convenient to consider the conductivity expressed in terms of the specific conductivity or $K \times 10^4$. And since the relationship between concentration and specific conductivity is (for the range under consideration) a straight line, a straight line representing this may be drawn on the same graph. As these conversions

are not always conveniently available to clinical men who may be interested in this method it is suggested that a chart similar to Fig. 2 be constructed. Any two points may be calculated from suitable tables and a straight line drawn between them. In Fig. 2 this line was constructed from the K_{20} values for NaCl equivalents of 0.5845 and 0.7014; namely, 0.009626 and 0.011446, respectively.

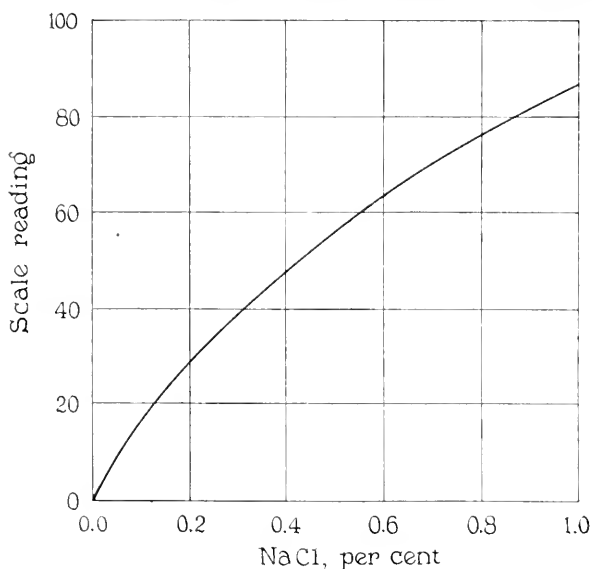


FIG. 1. Ionometer II. 110 volts D.C. at 20°C.

In Column 5 of Table I the difference between the values of $K \times 10^4$ calculated and observed for NaCl shows that the solutions used and the bridge readings were satisfactorily accurate.

The standardization curve shown in Fig. 1 corresponds to the type of curve to be expected from the equation $S_x = \frac{S_v R_v}{R_x K_2} = \frac{K_1 R_v}{R_x K_2}$ where S_v and S_x are the voltmeter scale readings, without and with the cell in series, R_x is the unknown resistance of the conductivity cell, and R_v is the resistance of the voltmeter. Since S_v and R_v are constants, $S_v R_v$ may be represented by K_1 , and R_v by K_2 .

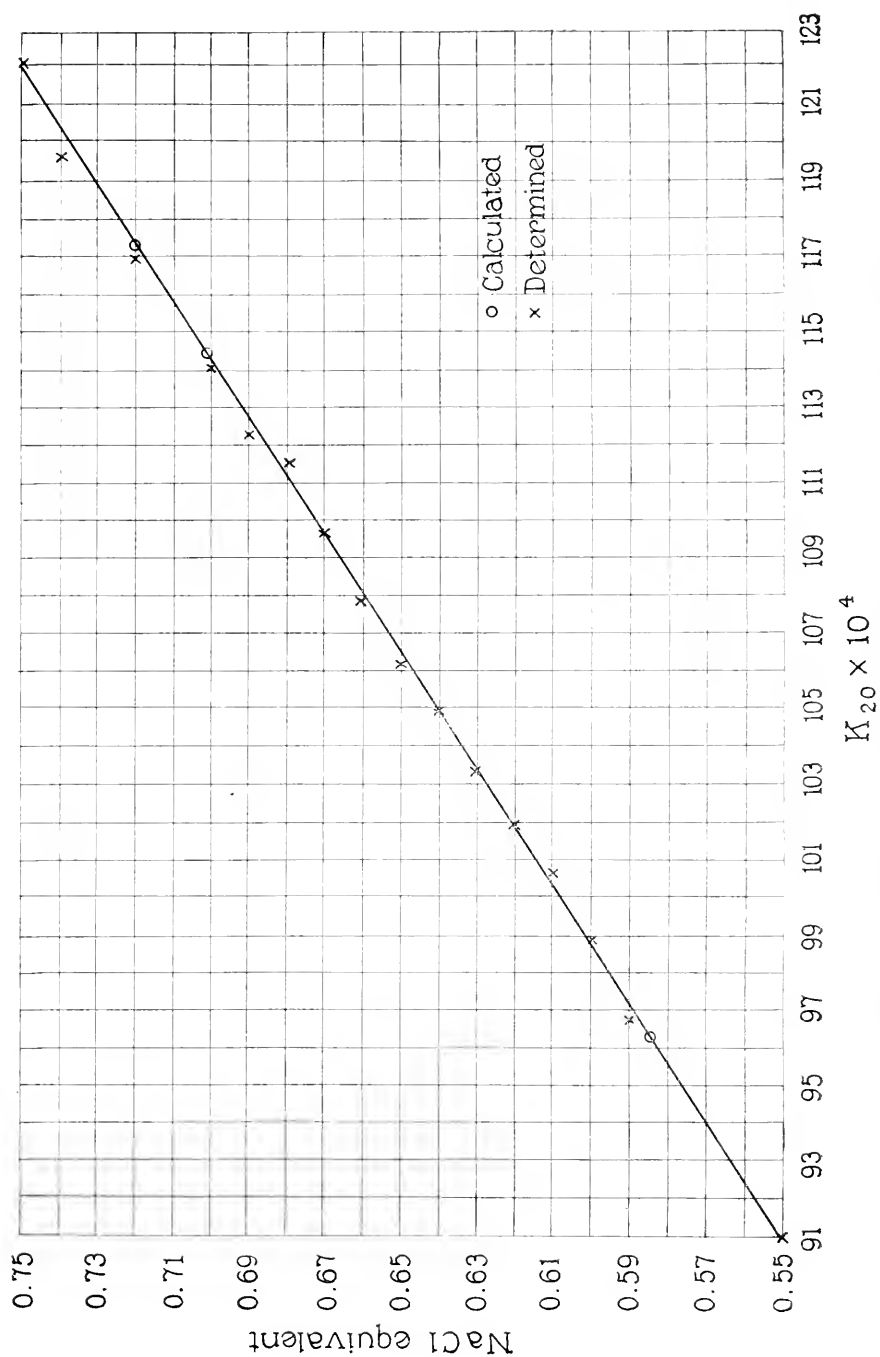


Fig. 2. Relation between NaCl equivalent and conductivity ($K_{20} \times 10^4$) at 20°C.

Table II gives a mean error determination. The mean error was determined by the formula $\mu = \sqrt{\frac{\sum d^2}{n-1}}$ where μ is the mean error, $\sum d^2$ is the sum of the squares of the variations from the average, and n equals the number of determinations. This is the formula used universally in Denmark, and is the formula used in the previous papers of this series.

TABLE I.
Standardization of Ionometer in Terms of NaCl Equivalents of Conductivity and of Specific Conductivity.

Columns 4 and 5 show the accuracy of the conductivity apparatus and of the solutions used.

NaCl solution used.	Ionometer scale reading.	$K_{20} \times 10^4$ Kohlrausch.*	$K_{20} \times 10^4$ Theoretical.†	Difference, Column 3 - Column 4 $K_{20} \times 10^4$
<i>gm. per 100 cc.</i>				
0.55	59.75	90.9	91.1	-0.2
0.59	62.25	96.7	97.1	-0.4
0.60	63	98.8	98.7	+0.1
0.61	64	100.6	100.2	+0.4
0.62	64.50	101.9	101.7	+0.2
0.63	65	103.3	103.3	0.0
0.64	65.75	104.9	104.8	+0.1
0.65	66.50	106.1	106.3	-0.2
0.66	67	107.8	107.9	-0.1
0.67	67.50	109.6	109.4	+0.2
0.68	68.25	111.4	111.0	+0.4
0.69	69	112.2	112.5	-0.3
0.70	69.75	114.0	114.1	-0.1
0.72	71	116.9	117.3	-0.4
0.74	71.75	119.6	120.4	-0.8
0.75	72.75	122.1	122.0	+0.1

* Corrected for the conductivity of the water used in making up the solution = 0.5×10^{-4} .

† Landolt, Boernstein, and Roth.

The next experiment was concerned with the influence of temperature on the readings. As shown by Table III the average change in conductivity per degree Centigrade between 25° and 15° is 2.06 per cent of the NaCl equivalent. It was found possible to keep the temperature constant within one-tenth of a degree Centigrade in our experiments; *i. e.*, to within 0.2 per cent of the reading.

TABLE II.

Mean Error of Ionometer Reading.

110 volts D.C. Ten determinations on a serum from a case of uremia; four readings for each determination

NaCl equivalent of conductivity.	Variation from average.
<i>per cent</i>	<i>per cent</i>
0.628	0.0016
0.628	0.0016
0.628	0.0016
0.628	0.0016
0.628	0.0016
0.620	0.0064
0.628	0.0016
0.624	0.0024
0.628	0.0016
0.624	0.0024
Average 0.6264	0.00244

Mean error = ± 0.0028 NaCl equivalent.

TABLE III.

Influence of Temperature on Ionometric Reading.

0.680 per cent NaCl used for experiment.

Temperature of bath.	NaCl equivalent of conductivity.	Difference per degree.
$^{\circ}\text{C.}$		
25	0.750	0.013
24	0.737	0.013
23	0.724	0.016
22	0.708	0.015
21	0.693	0.013
20	0.680	0.010
19	0.670	0.016
18	0.654	0.014
17	0.640	0.012
16	0.628	0.018
15	0.610	
Average		0.014

Average change per degree change of temperature = 2.06 per cent of value.

The results of conductivity with the ionometer were then compared with those obtained by a Kohlrausch apparatus. The platinized electrode cell of the bridge instrument was standardized by determining its resistance capacity with a 0.1 \times KCl solution, K_{20} taken as 0.01167. The KCl solution was tested by chloride titration and the results were as follows:

$$0.1 \times \text{KCl} = 0.7456 \text{ per cent KCl.}$$

Titration of triplicates.		
1	2	3
0.742	0.742	0.751
0.742	0.742	0.742
	0.732	0.751
Average = 0.744 or 99.8 per cent of the theoretical.		

Comparison of Methods.

With the calibration curve of the ionometer established and with the accuracy of the bridge apparatus verified, a series of comparisons of the two methods was carried out. Table IV gives the results of two such experiments in which KI and $(\text{NH}_4)_2\text{SO}_4$ were used. The maximum error was 1 per cent, the average error 0.5 per cent of the total.

Table V gives the results of a similar comparison in which sera were used. The average error in this series was 1 per cent with a maximum of 2.2 per cent. The conductivity as measured with the ionometer tended on the whole to be slightly lower than that measured with the bridge, but since the accuracy of scale reading, with the instrument used, is not greater than 1 per cent it is evident that the ionometric method is satisfactory for any investigation where an error of 1 per cent is permissible. This is certainly the case with the investigation of abnormal variation in conductivity in disease reported in the papers referred to above.

Influence of Protein, Urea, and Sugar on Conductivity.

It is well known that non-electrolytes added to a salt solution have a depressing influence on the conductivity of the salt solu-

TABLE IV.

Comparison between Ionometer and Kohlrausch Conductivity Determination.*

Salt.	Ionometer 20°C.		Kohlrausch 20°C.		
	¹ NaCl equivalent of conductivity.	$K_{20} \times 10^4$	² NaCl equivalents of conductivity.	$K_{20} \times 10^4$	Percentage difference.
Potassium iodide.					
<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>		<i>gm. per 100 cc.</i>		
1.20	0.530	87.8	0.531	88.0	+0.2
1.28	0.567	93.6	0.571	94.3	+0.7
1.36	0.605	99.4	0.599	98.4	-1.0
1.44	0.643	105.2	0.643	105.2	0.0
1.52	0.677	110.5	0.675	110.1	-0.3
Ammonium sulfate.					
0.68	0.612	100.5	0.615	101.0	+0.5
0.66	0.643	105.2	0.648	106.0	+0.8
0.76	0.674	110.0	0.675	110.1	+0.1

* As the absolute values were not important for our purpose these solutions were not prepared as carefully as the NaCl solutions used for standardization.

TABLE V.

Comparison of Conductivity Measurements by Ionometer and Kohlrausch Methods (Sera of Patients and Dogs).

Name.	Diagnosis.	Ionometer.		Kohlrausch.		Percentage difference.
		NaCl equivalent.	$K_{20} \times 10^4$	NaCl equivalent.	$K_{20} \times 10^4$	
T.G.	Pneumonia.	0.590	97.1	0.600	98.7	1.7
S.S.	Diabetes.	0.678	110.6	0.684	111.5	0.9
Fr.	"	0.624	102.3	0.636	104.2	1.9
S.A.	Pneumonia.	0.640	104.8	0.651	106.5	1.7
S.P.	Uremia.	0.628	102.9	0.630	103.2	0.3
Dog A.* ..		0.694	113.1	0.694	113.1	0
		0.707	115.2	0.718	117.0	1.6
Dog B....		0.713	116.1	0.718	116.9	0.7
		0.719	117.0	0.723	117.8	0.6
Dog C....		0.734	119.4	0.732	118.6	0.3
		0.730	118.8	0.726	118.2	0.5
Dog D....		0.691	112.6	0.699	114.0	1.2
		0.687	112.0	0.693	113.7	0.9
Dog E....		0.691	112.7	0.702	114.4	1.6
		0.691	112.7	0.706	115.0	2.2
Average		0.681	111.2	0.687	112.2	1.1

*Dog serum generally has a higher conductivity (and a higher chloride content) than human serum.

tion. Other investigators, notably Bugarszky and Tangl (4) and more recently, Palmer, Atchley, and Loeb (5,6) have measured the depressing influence of protein.

The influence of the most important non-electrolytes other than protein (glucose and urea) on *serum* conductivity can be dismissed as negligible since the depression is insignificant in concentrations which would correspond to a diabetes with 0.500 per cent blood sugar and to a uremia with 0.420 per cent blood urea. This is shown in Table VI.

In order to determine whether the protein depressive effect was the same with both methods, we compared the conductivity values of pure salt solution and of solutions containing the same salt concentration plus protein at a hydrogen ion concentration of pH 7.4. The protein content was determined by the refracto-

TABLE VI.
Influence of Non-Protein Non-Electrolytes on Conductivity (Ionometer).

Solution used.	NaCl equivalent of conductivity.
0.680 per cent NaCl	0.681
0.680 " " " + 0.400 per cent urea	0.681
0.680 " " " + 0.400 " " glucose	0.678

metric method, using an Abbé refractometer and Reiss values. The readings were corrected to the true index of redistilled water at 17.5°C. The correction equalled + 0.0032. The mean error of the method used was 0.1 per cent protein. The influence of urea and sugar up to the maximum concentration that can be expected in serum on the refractive index is not important, as will be seen from Table VII.

Two sources of protein were used in these experiments: dialyzed blood albumin and dialyzed human serum protein. Both were adjusted to pH 7.4. As will be seen in Table VIII the depression averages about 2.2 per cent of the total conductivity found per 1 per cent protein for both methods. This compares reasonably well with the value of 2.5 per cent found by Bugarszky and Tangl. This value was calculated from the conductivity values as follows:

$$d = \frac{(C_s + C_p - C_m) \cdot 100}{(C_s + C_p) \cdot P}$$

where C = conductivity of salt solution.

C_p = conductivity of protein solution.

C_m = conductivity of mixture.

P = protein in per cent in mixture.

d = per cent depression of total conductivity per 1 per cent protein.

TABLE VII.

Influence of Non-Protein Non-Electrolytes on Refractive Index of Serum.

Experiment No.	Solution.	Refractive index.	Remarks.
1	Distilled water.	1.32977	This corresponds to a change from 0.100 to 0.300 per cent in the blood. The difference in refractive index is 0.00035 or less than 0.2 per cent protein.
	Glucose, 0.200 per cent	1.33012	
	Distilled water.	1.32977	The difference from water in refractive index is 0.00032 or less than 0.2 per cent protein.
	Urea, 0.300 per cent	1.33009	
2	Distilled water.	1.32956	Difference in refractive index 0.00049 or less than 0.2 per cent protein.
	Acetone, 1 per cent.	1.33005	

It is assumed here that the correct total concentration equals the sum of the conductivity of the salt solution and of the protein solution. The depression effect of the protein in the term C_p itself is for simplicity neglected.

The true conductivity of the solution may be expressed as was pointed out by Bugarszky and Tangl

$$C_c = C_o \cdot \frac{100}{100 - (d \times P)}$$

where P = per cent protein.

C_c = corrected conductivity.

C_o = observed conductivity.

d = the per cent depression per 1 per cent protein.

The equation of Bugarszky and Tangl was based on the assumption that the depression was a function of the total conductivity

TABLE VIII.
Depressing Influence of Protein on Conductivity.

Experiment No.	Material used for proteins.	Solution.	Term used in equation.	Ionometer.		Kohlrausch.		Protein.	$\frac{d}{d}$ ionom-eter percent of total K per 1 K per 1 percent protein.	$\frac{d}{d}$ Kohl-rausch percent of total K per 1 K per 1 percent protein.
				NaCl equivalent.	$K \times 10^4$ at 20 C.	NaCl equivalent.	$K \times 10^4$ at 20 C.			
1	Solution of blood albumin acidified with acetic acid, dialyzed, brought to pH 7.4, and redialyzed. Final pH 7.4.	Dialyzed protein. 0.70 per cent NaCl. 0.70 per cent NaCl in dialyzed protein solution.	C_p		(1.6)*			per cent		
			C_s	0.697	113.6	0.698	113.7	3.0	2.06	1.79†
			C_m	0.662	108.1	0.668	109.1†			
2	Same as in Experiment 1.	Dialyzed protein. 0.70 per cent NaCl. 0.70 per cent NaCl in dialyzed protein solution.	C_p		(0.8)*			1.5	2.16	2.15
			C_s	0.697	113.6	0.698	113.7			
			C_m	0.678	110.7	0.678	110.8			
3	Same as in Experiment 1, concentrated in vacuum and redialyzed and readjusted to pH 7.4	Dialyzed protein. 0.70 per cent NaCl. 0.70 per cent NaCl in dialyzed protein solution.	C_p		(2.6)*			3.8	2.21	2.23
			C_s	0.700	114.1	0.701	114.2			
			C_m	0.654	106.9	0.651	106.9			

4	Dialyzed human serum prepared as in Experiment 1.	Dialyzed protein. 0.70 per cent NaCl. 0.70 per cent NaCl in dialyzed protein solution.	C_p C_s C_m	(2.1)* 114.1 108.1†	2.1 0.697 0.670	2.5 113.6 109.4	2.76‡	2.18
Average.....								
Total average.....								
2.29								
2.19								

* Because of the inaccuracy of the ionometer reading at this dilution the Kohlrausch value has been used for the factor C_p with both methods.

† The Kohlrausch reading in Experiment 1 and the ionometer reading in Experiment 4 are probably wrong. If we take the averages of the remaining figures we get d ionometer = 2.14 and d bridge = 2.19 or an average d of 2.17 which may be rounded off to the value of 2.2.

of the solution (without protein) as well as of the protein concentration. The fact that the values for conductivity in the above experiment as well as those of Palmer, Atchley, and Loeb are a linear function of one protein concentration does not indicate whether the depression is a function also of the electrolyte concentration or whether there is a definite absolute decrease in

TABLE IX.

To Show Accuracy of Protein Correction Factor at Various Concentrations of Protein.

Experiment No.	Solution used.	<i>P</i> Protein.	<i>C_o</i> Observed NaCl equivalent of conduc- tivity.	Actual <i>C_c</i> Corrected NaCl equivalent of conduc- tivity.	<i>C_c</i> Expected NaCl equivalent of mixture.	Differ- ence.
		<i>per cent</i>				
1a	Serum.	8.2	0.647	0.789		
	NaCl solution.	0	0.772	0.772		
	Serum + equal volume NaCl solution.	4.1	0.711	0.781	0.781	0.00
1b	Serum + equal volume distilled water.	4.1	0.359	0.395	0.394	+0.001
2a	Serum.*	8.2	0.640	0.780		
	NaCl solution.	0	0.790	0.790		
	Serum + equal volume NaCl solution.	4.1	0.712	0.782	0.785	-0.003
2b	Serum + equal volume distilled water.	4.1	0.352	0.387	0.390	-0.003

* Serums 1 and 2 were from different subjects.

conductivity for each increment of protein, independent of the electrolyte concentration. The first assumption leads to the equation,

$$C_c = \frac{C_o \times 100}{100 - (d \times P)}$$

and the second, to the equation

$$C_c = C_o + (d \times P)$$

The results of Bugarszky and Tangl indicate that the first assumption is correct and its correctness is verified by the experiment recorded in Table IX where concentrations both of protein and salt are varied. The equation $C_o = \frac{C_c \times 100}{100 - (d \times P)}$ holds, while the equation based on the second assumption does not.

SUMMARY.

1. The ionometric method of Christiansen for measuring conductivity of electrolytes ordinarily agrees with the Kohlrausch method to within 1 per cent and with a maximum difference of 2 per cent.

2. Urea or glucose in the highest concentration possible in serum causes negligible depression of conductivity and negligible change in refractive index.

3. The depression of conductivity due to protein has been found to be the same with the ionometric and with the Kohlrausch method. Our average value of 2.2 per cent per 1 per cent protein agrees with the values reported by other observers. Serum conductivity values should be corrected to a protein-free basis. The refractometer method of determining the protein concentration is adequate for this purpose.

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A MICRO METHOD FOR THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF WHOLE BLOOD.

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The electrometric and colorimetric methods for determining the blood reaction in use at present require such quantities of blood that they are not applicable for use with small laboratory animals.

The method described below, requiring only small quantities of blood, is a slight modification of the method recently described by Cullen.¹

Experiment.

.30 drops of a 0.03 per cent solution of phenol red are added to 50 cc. of a 0.9 per cent solution of sodium chloride and adjusted to pH 7.3. 5 cc. portions of this solution are placed in tubes with a diameter of 16 mm. and covered with paraffin oil.

The blood is drawn from the heart or a vein directly into a 1 cc. pipette graduated to hundredths, by attaching a needle with a short rubber tube to the pipette.

The needle and rubber are then detached and 0.25 cc. of the blood is run under the oil into one of the tubes containing the saline indicator solution. The blood and solution are thoroughly mixed by stirring carefully with a clean glass rod.

The tube is centrifuged for 10 minutes, completely throwing down the red corpuscles and is then placed in a comparator block and the pH determined by matching to the nearest color standard and applying corrections as described by Cullen. The standard color tubes are 16 mm. in diameter and contain 5 cc. of Sörenson's standard phosphate solutions ranging in steps of 0.05 pH from pH 7.00 to 7.80.

For the comparison pH determinations were made simultaneously on the same sample of blood, using both the above modification and the original method as described by Cullen.¹

¹ Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

Comparison of pH Values as Determined by the Two Methods.

No.	Whole blood.	Plasma.	Deviation.
Rabbits (arterial blood).			
1	7.29	7.29	0.00
2	7.29	7.30	-0.01
3	7.32	7.34	-0.02
4	7.42	7.42	0.00
5	7.29	7.31	-0.02
6	7.49	7.48	+0.01
7	7.32	7.35	-0.03
8	7.53	7.53	0.00
9	7.48	7.50	-0.02
10	7.48	7.46	+0.02
11	7.41	7.41	0.00
12	7.46	7.47	-0.01
13	7.42	7.42	0.00
14	7.43	7.41	+0.02
15	7.46	7.43	+0.03
16	7.45	7.43	+0.02
17	7.43	7.41	+0.02
18	7.26	7.25	+0.01
19	7.33	7.32	+0.01
20	7.30	7.29	+0.01
21	7.32	7.33	-0.01
22	7.33	7.33	0.00
23	7.31	7.32	-0.01
24	7.32	7.33	-0.01
Rabbits (venous blood).			
1	7.29	7.30	-0.01
2	7.33	7.33	0.00
3	7.34	7.37	-0.03
4	7.35	7.37	-0.02
5	7.32	7.33	-0.01
6	7.29	7.30	-0.01
Guinea pigs (arterial blood).			
1	7.42	7.44	-0.02
2	7.46	7.46	0.00
3	7.44	7.44	0.00
4	7.45	7.48	-0.03
5	7.40	7.40	0.00
6	7.36	7.35	+0.01

Comparison of pH Values as Determined by the Two Methods—Concluded.

No.	Whole blood.	Plasma.	Deviation.
Guinea pigs (arterial blood)—Concluded.			
7	7.31	7.31	0.00
8	7.37	7.35	+0.02
9	7.36	7.36	0.00
10	7.33	7.34	-0.01
Guinea pigs (venous blood).			
1	7.38	7.39	-0.01
2	7.32	7.34	-0.02
3	7.46	7.44	+0.02
4	7.49	7.49	0.00
5	7.32	7.32	0.00
Human (venous blood).			
1	7.31	7.34	-0.03
2	7.29	7.30	-0.01
3	7.35	7.35	0.00

The pH as determined by the two methods agrees in most cases, the maximum deviation being 0.03 pH. The values reported above were all obtained at room temperatures between 20 and 24°C. The values of the rabbit and human bloods are corrected to electrometric pH_{38° ; i.e., $\text{pH}_{38^\circ} = \text{pH}_{\text{colorimetric } 20^\circ} - C$.

This method can be used in studying the changes of reaction in small laboratory animals during a course of experiments without affecting the animals by loss of blood. In addition, the total CO_2 content of the blood may also be determined, using 0.2 cc. of the excess blood in the pipette, by Van Slyke's method.²

SUMMARY.

A method is described for determining the reaction and the total CO_2 content of the blood using 0.6 cc. of the whole blood instead of plasma, thus rendering it possible to make repeated observations on small laboratory animals.

² Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.

A QUANTITATIVE COLOR REACTION GIVEN BY ADRENALIN AND URINE.

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(Received for publication, March 12, 1923.)

The assay of adrenalin for commercial purposes is still performed physiologically and there appears to be no satisfactory quantitative chemical determination for the substance in either blood or urine. The Folin, Cannon, and Denis (1) assay on the gland, based on the property of adrenalin to give a blue color quantitatively with phosphotungstic acid, is not applicable to blood or urine. A complement fixation test has been applied by Polito and Covelli (2) for the diagnosis of hyperfunction of the suprarenal glands, in which an alcoholic extract of these glands is used as an antigen. The results were not conclusive. Ehrmann (3), Meltzer and Auer (4), Wessely (5) and others, found that when epinephrine is injected subcutaneously or applied to a frog's eye dilation of the pupil is produced. Ehrmann suggested this as a test in blood, but he overlooked the fact that in blood there are other substances which will produce the same reaction. The Goetsch test (6), a purely clinical procedure, in use, does not give consistent results.¹

The color reaction here described depends on the fact that epinephrine reacts with sulfanilic nitrous acids in the presence of an alkali (ammonia) to give a red color. The phenols present in urine and which cannot be removed also give the color. This necessitates the running of two determinations simultaneously, one

* Specimens were obtained through the courtesy of Dr. Herbert J. Wiener.

¹ Upham, R., and Blaivas, A. J., Personal communication on unpublished data.

with the addition of an iron suspension to destroy the adrenalin. The phenols are not affected by the iron. The amount of adrenalin, or of substance reacting like adrenalin, is estimated by the decrease in the color of the one containing the iron.

In order to show that the color is produced quantitatively the following experiments were performed.

Experiment 1.—A solution of adrenalin was made containing 1 mg. per cc., by dissolving the base in a 2 per cent solution of hydrochloric acid, made from freshly distilled water. Varying quantities of this solution were pipetted into 10 cc. volumetric flasks; exactly 5 cc. of the sulfanilic acid were added, followed by exactly 1 cc. of the nitrite solution; water was then added to the mark, the flasks were shaken and the contents transferred to

TABLE I.

Tube No.	Amount of adrenalin	Tube 1 used as standard and set at.	Reading.
	<i>cc.</i>	<i>mm.</i>	<i>mm.</i>
1	0.0		
2	0.1	20.0	18.0
3	0.2	20.0	16.0
4	0.3	20.0	14.0
5	0.4	20.0	12.0
6	0.5	20.0	10.0
7	0.6	20.0	7.9
8	0.7	20.0	6.0
9	1.0	20.0 (No. 6 used as standard.)	10.0

test-tubes. 3 cc. of ammonia were added. The tubes were quickly stoppered and set aside for 15 minutes, then compared in a colorimeter. The results are given in Table I.

Experiment 2.—1 and 2 cc. of the same solution were added to urine as follows: First, 98 cc. of urine plus 2 cc. of distilled water were carefully pipetted into a 300 cc. Erlenmeyer flask. To a second flask, 98 cc. of the same sample plus 1 cc. of the adrenalin solution and 1 cc. of water were pipetted; and to a third, 98 cc. of the same urine plus 2 cc. of adrenalin solution. To each were added 50 cc. of water (to conform with the test); next, 20 cc. of the sulfanilic acid, then 5 cc. of the nitrite solution were added, followed by 10 cc. of ammonia. Flasks were quickly stoppered, shaken, and compared after standing for 15 minutes. The results are given in Table II.

Experiment 3.—The precipitating reagents were tested for the purpose of making certain of the recovery of the added adrenalin after treatment with them, and also of its destruction by the iron suspension. First, 297 cc. of

urine to which 3 cc. of *distilled water* had been added were treated as outlined in the method. To another 297 cc. of the same specimen 3 cc. of the *adrenalin solution* were added and similarly treated. The results are given in Table III.

TABLE II.

Urine used as standard set at.	1 mg. adrenalin added to urine.		2 mg. adrenalin added to urine.	
	Reading.	Adrenalin recovered.	Reading.	Adrenalin recovered.
<i>mm.</i>	<i>mm.</i>	<i>mg.</i>	<i>mm.</i>	<i>mg.</i>
20.0	15.0	1.0	10.0	2.0
20.0	15.0	1.0	10.0	2.0
20.0	15.0	1.0	10.0	2.0
20.0	15.1	0.98	10.0	2.0
20.0	15.0	1.0	10.1	1.98
20.0	15.2	0.96	10.0	2.0
20.0	15.0	1.0	10.1	1.98
20.0	15.0	1.0	10.0	2.0

TABLE III.

Specimen No.	Adrenalin content of urines used.			Same urines with 1 mg. adrenalin added.		
	Flask A set at.*	Reading of Flask B.†	Amount of adrenalin present.	Flask A set at.*	Reading of Flask B.†	Amount of adrenalin present + recovered.
	<i>mm.</i>	<i>mm.</i>	<i>mg.</i>	<i>mm.</i>	<i>mm.</i>	<i>mg.</i>
1	20.0	18.0	0.40	20.0	13.1	1.38
2	20.0	18.1	0.38	20.0	13.2	1.36
3	20.0	19.0	0.20	20.0	14.0	1.20
4	20.0	17.2	0.56	20.0	12.2	1.56
5	20.0	17.5	0.50	20.0	12.5	1.50
6	20.0	15.0	1.00	20.0	10.1	1.98
7	20.0	18.0	0.40	20.0	13.0	1.40
8	20.0	17.2	0.56	20.0	12.2	1.56
9	20.0	19.0	0.20	20.0	14.2	1.16
10	20.0	14.9	1.02	20.0	9.9	2.02

* Adrenalin destroyed.

† Reading of adrenalin and phenol.

Method for Urine.

Reagents.—The following reagents are used:

Lead acetate, 25 per cent solution; U. S. P. grade may be used in making this solution; it should be filtered.

Ammonium sulfate, 15 per cent solution; the crystals used must be c. p. and free from iron (0.0002 per cent). Merek's quality was found to be suitable for this purpose.

Iron suspension. This is made by dissolving 0.025 gm. of ferric chloride in water and making up to 1 liter. This must be standardized by adding 20 cc. of it to water, then adding the color-producing reagents and making up to 180 cc. Upon comparison against a blank of color-producing reagents only, it should

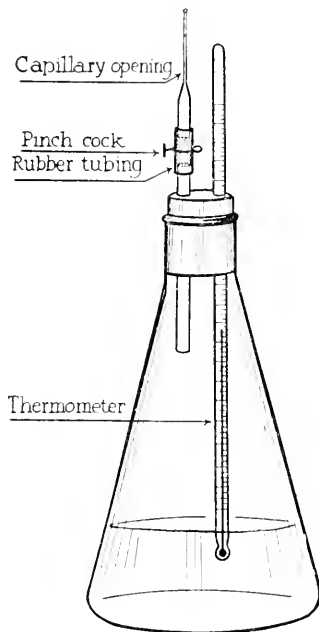


FIG. 1.

not give any color. Slight dilution, however, will overcome a possibility.

Sulfanilic acid. 4.0 gm. of sulfanilic acid crystals are mixed with 90 cc. of 37 per cent hydrochloric acid, sp. gr. 1.19, in a liter flask and distilled water is added to the mark. Allow to stand; shake occasionally. The solid dissolves slowly but completely.

Sodium nitrite, a 1 per cent solution.

Ammonium hydroxide, 28 per cent.

Apparatus. The only special apparatus (Fig. 1) is a 300 cc. Erlenmeyer flask fitted with a capillary opening, pinch-cock arrangement, and a Centigrade thermometer. Another 300 cc. rubber stoppered Erlenmeyer flask is also used. Marking pencils are not to be used to pronounce obliterated thermometer markings. A rubber band or dental floss answers this purpose.

Technique.—To 300 cc. of urine in a precipitating flask add 37.5 cc. of the lead acetate solution and 37.5 cc. of the ammonium sulfate; stir with a glass rod, and let stand for 15 minutes. The precipitate settles quickly. Decant the supernatant fluid through any good quality filter paper. Centrifuge may be employed. Filtrate must be free of any sediment. Two exactly 125 cc. quantities of the filtrate are transferred to Flasks A and B. To Flask A add 20 cc. of the iron suspension after shaking the latter; note temperature and heat slowly over a low flame, rotating flask until 50°C. is reached. Cool under the tap to the observed temperature and clamp the capillary tube. (Add reagents immediately to both flasks after cooling.) To Flask B add 20 cc. of distilled water. To each add exactly 20 cc. of sulfanilic acid, exactly 5 cc. of sodium nitrite, and 10 cc. of ammonia; shake; let stand for 15 minutes. Compare one with the other in either a Duboseq or a Kober colorimeter. Explanation of the readings can be shown best by the following.

A standard phenol solution² was made—1 cc. equivalent to 1.567 mg. of pure phenol. 25.55 cc. quantities of this solution equal to 40.036 mg. of phenol were added to various Erlenmeyer flasks. To these were also added 0.5, 1.0, 1.5, and 2.0 cc. quantities of a freshly prepared 0.1 per cent adrenalin solution. They were in turn then treated as per the above method and made up to 180 cc. The means of several readings were:

0.5 cc. of 0.1 per cent adrenalin	2.5 mm. more dense than phenol solution.
1.0 " " 0.1 " " " "	5.0 " " " " " "
1.5 " " 0.1 " " " "	7.5 " " " " " "
2.0 " " 0.1 " " " "	10.0 " " " " " "

² Standardization of phenol. A phenol solution was prepared in 0.1 N hydrochloric acid which contained approximately 1 mg. of crystallized phenol per 1 cc. I transferred 25 cc. of this solution to a 250 cc. flask, added 50 cc. of 0.1 N sodium hydroxide, heated to 65°C., added 25 cc. of 0.1 N iodine solution, stoppered flask, and let stand for 30 minutes at room temperature. I then added 5 cc. of concentrated hydrochloric acid and titrated the excess of iodine with 0.1 N thiosulfate solution. Each cubic centimeter of 0.1 N iodine solution corresponds to 1.567 mg. of phenol.

TABLE IV.

Patient.	Sex.	Diagnosis.	Specimen.	Albumin.	Sugar.		Readings.		Adrenalin per 100 cc. urine equivalent to color observed.
					Benedict's modification.	Nylander's.	Flask A.	Flask B.	
H.F.	M.	Normal.	Morning.	0	0		20.0	19.0	0.2
J.R.	"	" (low blood pressure).	24 hr.	0	Trace.	Trace.	20.0	14.9	1.02
F.E.	"	"	24 "	0	0		20.0	19.0	0.2
A.C.	"	" (very nervous).	Casual.	0	Trace.	Trace.	20.0	16.5	0.7
J.H.	"	"	24 hr.	0	0		20.0	18.0	0.4
R.A.	F.	Diabetes mellitus (treated).	24 "	0	0		20.0	18.0	0.4
M.T.	M.	" "	Casual.	0	0		20.0	20.0	None.
M.R.	F.	Renal calculi?	24 hr.	0	Trace.	Trace.	20.0	17.9	0.42
A.M.	"	"	24 "	Trace.	0.7	Present.	20.0	17.6	0.48
M.M.	M.	"	24 "	0	0.9	"	20.0	20.0	None.
H.B.	F.	"	Casual.	0	0		20.0	19.3	0.14
I.E.	M.	Glycosuria.	Morning.	0	Trace.	Trace.	20.0	15.0	1.0
H.T.	"	Diabetes mellitus.	24 hr.	0	2.4	Present.	20.0	15.0	1.0
R.W.	F.	"	24 "	0	3.1	"	20.0	20.0	None.
C.H.	"	"	24 "	0	0.5	"	20.0	14.5	1.1
B.F.	"	Glycosuria.	24 "	0	Trace.	Trace.	20.0	16.5	0.7
H.M.	M.	"	24 "	0	"	"	20.0	15.5	0.9
S.B.	"	Nephritis (active person).	24 "	+ casts.	"	"	20.0	15.0	1.0
M.P.	"	"	24 "	++++	0		20.0	19.0	0.2

* Benedict's (8) second modification was used here.

Tests were conducted in a boiling water bath for 5 minutes.

R.D.	M.	Nephritis (chronic).	24 hr.	++ casts.	0	Trace.	20.0	19.0	0.2
J.W.	"	"	24 "	Trace, casts.	Trace.	Trace.	20.0	16.7	0.66
J.L.	F.	"	24 "	++ ++ ++ casts.	0	0	20.0	19.3	0.14
M.P.	"	(chronic).	Morning.	++ ++ ++ casts.	Trace.	Trace.	20.0	16.8	0.64
H.T.	"	"	24 hr.	Trace.	0	0	20.0	19.0	0.2
A.W.	M.	" (heart condition).	24 "	Trace, casts.	Trace.	Trace.	20.0	16.3	0.74
V.M.	F.	" jaundice.	24 "	0	"	"	20.0	16.7	0.66
A.F.	"	Hypertension.	Casual.	0	0	0	20.0	18.0	0.4
G.F.	M.	"	24 hr.	Trace.	Trace.	Trace.	20.0	17.0	0.6
S.D.	"	"	24 "	0	0	0	20.0	18.0	0.4
W.E.	"	Gastrointestinal.	24 "	0	Trace.	Trace.	20.0	16.8	0.64
P.B.	F.	Albuminuria.	24 "	++ ++ ++	0	0	20.0	20.0	None.
J.B.	"	Neurasthenia.	24 "	++ casts.	Trace.	Trace.	20.0	14.5	1.1
W.L.	M.	Pneumonia.	Casual.	0	"	"	20.0	15.0	1.0
S.W.	"	Carcinoma kidney.	24 hr.	++ ++ ++	0	0	20.0	15.0	1.0
S.H.	F.	Albuminuria.	Morning.	0	0	0	20.0	19.0	0.2
B.S.	"	Arthritis.	Casual.	0	0	0	20.0	19.0	0.2
R.W.	M.	Neurasthenia.	24 hr.	0	Trace.	Trace.	20.0	16.5	0.7
J.K.	F.	"	24 "	0	"	"	20.0	17.0	0.6

Calculation.—125 cc. of the filtrate are equivalent to 100 cc. of the original urine. Comparison is best made by setting the contents of Flask A at 20.0 mm. and comparing those of Flask B to it. Since 1 mg. of adrenalin gives a reading of 5 mm., it follows that the reading multiplied by 0.2 gives the amount present.

For example: Reading is 2.0 mm. difference, $2.0 \times 0.2 = 0.4$ mg. per 100 cc. urine.

DISCUSSION.

No explanation can be offered at this time for the use of the lead acetate as there are no available data specifying the urinary constituents precipitated by it. In some cases, however, higher results were obtained where it was omitted. It is necessary at this point to remove all the lead, which is accomplished by the use of the ammonium sulfate. In regard to the destruction of the epinephrine, all the oxidizing agents were found to be objectionable, but iron lends itself admirably well to meet this end, since slight traces are absolutely destructive to the base and the amount used does not add to or interfere with the color produced.

Careful tests were conducted on the other urine constituents, organic, inorganic, and pathological, to establish definitely that they do not give a reaction with these reagents. The tests were conducted, both in test-tubes and by the addition of each one separately to urine. Acetone and some of the amino-acids and phenols, as already shown, were the only ones found to give this red color. These have no bearing on the determination, however, since none is affected by either the iron suspension or the heat as found by exhaustive experimentation. These experiments were quantitative in nature, carried out as per test.

A point of possible significance in Table IV is the frequent coincidence of relatively high adrenalin color reactions and slight Benedict sugar tests in non-diabetic urine. Adrenalin reduces Benedict's, Fehling's, and Nylander's solutions. It seems possible that slight reductions occasionally observed in glucose-free urines may be due to adrenalin present. It may also be mentioned here that when adrenalin is treated with a saturated solution of picric acid and sodium carbonate, then heated as per the Lewis and Benedict (7) blood sugar determination, the same red color typical of sugar is produced.

A few cases, taken from some 250, are presented in Table IV to give some idea of the amount of substance giving the adrenalin color reaction present in normal urine and in some pathological conditions. The greater number of these specimens were 24 hour collections.

Normal urines were found to give a usual reaction equivalent to 0.2 to 0.4 mg. per 100 cc.

The diagnoses of the cases cited were not made by the writer.

CONCLUSION.

The color reaction given by pure solutions of adrenalin with sulfanilic and nitrous acids is quantitatively proportional to the adrenalin concentration.

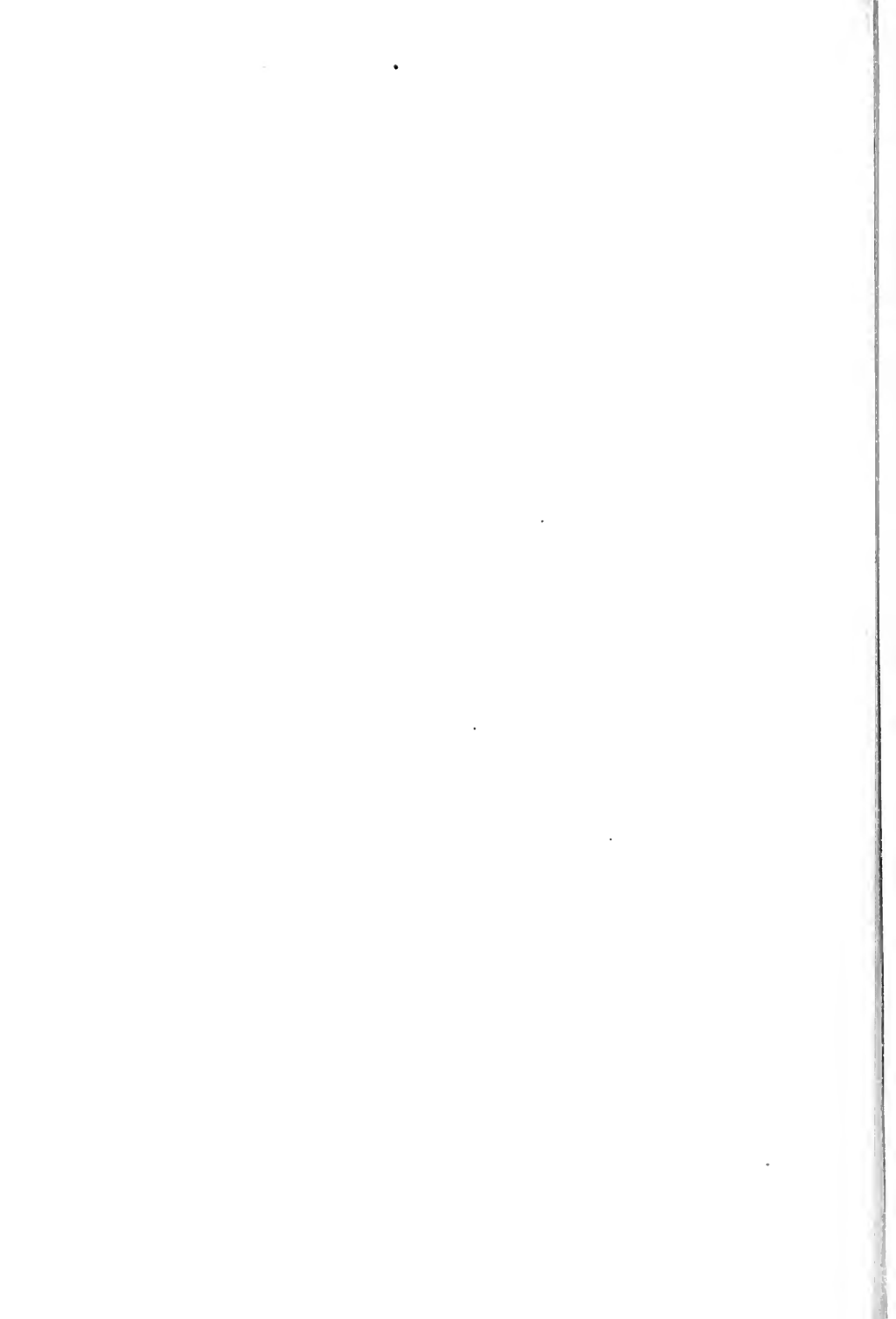
After treatment with ferric chloride, adrenalin no longer gives this reaction. None of the urinary constituents tested has given a positive reaction before ferric chloride treatment and a negative reaction after it. It appears possible that the urinary constituent giving the color before, but not after, the ferric chloride treatment is adrenalin.

Determinations have been made of this constituent in a number of normal and pathological urines.

I wish to thank Parke, Davis and Co., New York, who very generously supplied all the adrenalin used in this work.

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NITROGEN DISTRIBUTION IN GLOBIN.

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In endeavoring to isolate any single component from a naturally occurring mixture of proteins, one is almost certain to be embarrassed by the lack of some safe yet convenient criterion which shall guarantee the completeness of the separation. The writers lately experienced this difficulty in attempting to obtain preparations of globin which should be demonstrably uncontaminated by the other proteins of the blood. They had barely encountered the problem when its solution seemed to be offered. Fürth and Lieben (1) reported in 1920 the entire absence of the tryptophane radicle from the molecule of hemoglobin. Here appeared to be the desired criterion. The other blood proteins are known to be fairly rich in tryptophane, so that the problem apparently resolved itself into one of purifying hemoglobin, or its globin component, until it gave a negative glyoxylic reaction.

We have already reported elsewhere the failure of the expectations thus raised (2). It has been found that not even five recrystallizations of hemoglobin made any appreciable difference in the intensity of the glyoxylic reaction as given either by the pigment itself or by the globin prepared from it. Not only is this so, but the method of tryptophane estimation based by Fürth and Nobel (3) upon the Voisenet reaction—the very method the use of which had led Fürth and Lieben to conclude that hemoglobin is free from tryptophane—indicated in our hands the presence in globin of 2.6 per cent of that amino-acid. This proportion was not in the least diminished by successive purifications. We were forced to the conclusion—quite in accord with earlier belief (4, 5)—that tryptophane is an integral part of the globin molecule; and could only suppose that Fürth and Lieben had been misled by the use of globin solutions altogether too dilute to give the Voisenet reaction.

This and other experiences with the tryptophane method of Fürth and Nobel led us to doubt seriously the reliability of quantitative data obtained by its application. When, therefore, just as we completed our work with it, Folin and Looney (6) described another and apparently better method of determination, a method based upon a different color reaction and capable moreover of convenient combination with a quantitative procedure for tyrosine, it seemed to us worth while to review the problem again. With the aid of this newer method we have now determined the tryptophane and tyrosine content of two series of globin preparations, and have, we believe, settled fairly decisively the proportion of these amino-acids yielded by the pure protein. We have also taken occasion to determine by the method of Van Slyke the general distribution of nitrogen in the globin molecule.

Meanwhile, our original conclusion has been independently confirmed in Fürth's own laboratory. Kiyotaki (7), still using the method of Fürth and Nobel, but observing certain precautions neglected by Fürth and Lieben, estimates the tryptophane content of globin at the rather high figure of 3.6 per cent. He has also determined the tyrosine content, which he places at 3.5 to 4 per cent. Since in each determination his result as well as his method is different from ours, the presentation of our data would still seem to be worth while.

Globin Preparations.

First Series.—A large quantity of crystalline oxyhemoglobin was obtained from horse blood by the method of Zinoffsky (8). The preparation was recrystallized four times. Of each of the first four crops of crystals a portion was reserved. From each reserved portion, and from the whole of the fifth and final crop, globin was separated by a slight modification of the method of Robertson (9). Five successive specimens of globin, of presumably increasing purity, were thus obtained. The first and fourth were somewhat contaminated with hematin; but the second, third, and fifth were nearly colorless.

Second Series.—The second series of globin preparations differed from the first in two respects. The original solution of hemoglobin, obtained from the laked corpuscular mass, was treated with alumina cream according to the method of Marshall and Welker (10);

and the number of recrystallizations was reduced to two. The series included, therefore, only three specimens. Of these the first and second were rather pigmented; the third was practically white.

The nitrogen content of the globin preparations, determined by the method of Kjeldahl, varied between 16.76 and 16.93 per cent. The average found was 16.86, which agrees with the value 16.89 reported by Schulz (11). The variations, such as they were, were quite irregular, and in themselves gave no evidence of increasing purity in successive fractions.

Determinations of Tryptophane and Tyrosine.

1 gm. lots of the eight preparations of globin, each of which had been dried for 48 hours *in vacuo* over sulfuric acid, were hydrolyzed with baryta, and assayed for tryptophane and tyrosine by the method of Folin and Looney (6). Each determination was carried out on duplicate samples of the product of hydrolysis, the volume taken—4 to 5 cc.—being always such as would yield color depths approximately equal to those of the standards.

The results are reported in Table I, in which is shown also for each case the indicated molecular ratio of tyrosine to tryptophane.

TABLE I.

Source of globin.		Dry globin.		Molecular ratio Tyrosine Tryptophane
		Trypto- phane.	Tyro- sine.	
		<i>per cent</i>	<i>per cent</i>	
First series.	First crystallization.....	1.93	3.23	1.9
	Second "	1.99	3.57	2.0
	Third "	2.51	3.97	1.8
	Fourth "	2.49	3.96	1.8
	Fifth "	2.48	4.13	1.9
Second series.	First "	2.56	4.10	1.8
	Second "	2.61	4.64	2.0
	Third "	2.62	4.63	2.0

Discussion of the Tryptophane and Tyrosine Determinations.

It is at once apparent from the data that no *diminution* of tryptophane content is effected by successive attempts at purifica-

tion. Whether the *increase* observed in the earlier samples of each series, and the higher maximum shown by the second, have a real significance is not quite certain. The differences seem, especially in the first series, to be rather beyond the experimental error of the method; but the fact that they are paralleled by nearly proportional changes in tyrosine content makes them difficult to explain by simple contamination of the first fractions with at any rate the *serum* proteins. Serum globulin (ox) contains, according to Folin and Looney, 6.7 per cent of tyrosine and 2.28 per cent of tryptophane. A highly purified specimen of serum albumin (horse), to which we applied Folin and Looney's procedure, yielded values of 4.63 per cent for tyrosine and 1.79 per cent for tryptophane. The gradual removal of serum proteins would therefore be expected to raise the tryptophane content of the preparations, but to depress their tyrosine content. Actually the proportion of *each* amino-acid is seen to increase regularly to a maximum, while the molecular ratio remains nearly constant. Possibly an admixture of stroma proteins, regarding the composition of which we know next to nothing, may be responsible for the low tyrosine content of the early fractions.

Taking the analytical results at their face value, it would seem that to obtain a globin of constant composition by simple recrystallization of oxyhemoglobin, three crystallizations at least are necessary; whereas, when a preliminary treatment with alumina cream is employed, two will suffice. This is a confirmation of the claims made (10) for the efficacy of alumina cream in purifying oxyhemoglobin from other colloids.

There is, it must be admitted, a discrepancy between the two series in respect to the ultimate values, especially for tyrosine, to which they rise. While we cannot at present offer an adequate explanation, we feel that the second series deserves the greater confidence, and would therefore put the tryptophane yield of pure globin at 2.61 per cent and its tyrosine at 4.63. This would attribute to each amino-acid the same proportion—2.12 per cent—of the total globin nitrogen.

At these values we get a molecular ratio of tryptophane to tyrosine that is exactly 1:2. On the assumption that this implies the presence in the globin molecule of two tryptophane and four tyrosine radicles, we may then calculate the molecular

weight of globin to be from 15,630 to 15,640. This agrees surprisingly with the 15,274 deduced by Osborne from the sulfur content (12).

The tryptophane value of 2.6 per cent, yielded by the method of Folin and Looney, is identical with that which we formerly obtained (2) by the method of Fürth and Nobel; but the latter in the hands of Kiyotaki (7) gave much higher figures—from 3.1 to 4.0, with an average of 3.6. A higher value by the Fürth-Nobel procedure is the general rule (6), and it may be that the identity of our two results is the chance outcome of the conditions under which the earlier determinations were made. Kiyotaki shows, what our own experience had already suggested, that the result of a Fürth-Nobel determination is greatly dependent upon the concentration of the protein analyzed. We suspect that it is affected by still other factors. We have observed, for instance, that the Fürth-Nobel procedure gives a far deeper and at the same time redder color when applied to glycyl-tryptophane than when applied to an equivalent quantity of free tryptophane;¹ and that the few instances where the Folin-Looney result is not decidedly lower than the other—casein (6), serum albumin (1.8 per cent by Folin and Looney, 1.4 by Fürth and Nobel (7)), and globin (if the comparison is limited to our own determinations)—include only proteins that are free from glycocoll. These observations are too limited in number to be decisive; but they certainly suggest that the Voisenet color reaction is affected, both as to hue and as to intensity, by the mode of combination of tryptophane in the protein molecule. This suspicion is not calculated to increase one's confidence in any method of which it forms the basis.

The tyrosine content of globin was determined by Kiyotaki in two ways; (1) by a bromine titration, and (2) by a colorimetric application of the diazo reaction. Neither method gave very consistent results, the percentage found varied from 2.2 to 5.5. The figure 3.5 to 4.0 is proposed as a probable approximation.

From his data Kiyotaki calculates that if globin has a molecular weight of about 15,000, it should contain 3 molecules each

¹ Proteins also give a redder color than pure tryptophane, a fact already commented upon by Lüscher (Lüscher, E., *Biochem. J.*, 1922, xvi, 556), who produces, moreover, fairly conclusive evidence that values obtained by the Fürth-Nobel method are 30 to 60 per cent too high.

of tyrosine and of tryptophane. We feel that the greater consistency of our own results, together with the pains taken to prove the purity of our globin, make our estimate the more probably correct one.

General Distribution of Nitrogen in Globin.

The preparation of globin used for the Van Slyke analysis had been obtained from oxyhemoglobin, twice crystallized after treatment with alumina cream. It had a light brown color, and included, therefore, doubtless a trace of hematin. Two portions were separately hydrolyzed with 20 per cent hydrochloric acid, the one (designated A in Table II) by heating in a bath of boiling water for 48 hours, the other (B) by boiling over a free flame for 30 hours. Table II shows the results obtained with aliquot portions of each hydrolysate. The original analytical data have been corrected for the solubility of the phosphotungstates of the bases. No correction was applied for cystine, for like Van Slyke in his analysis of hemoglobin (13) we found, even in the much larger quantities with which we were dealing, no evidence of the presence in the base fraction of any appreciable quantity of that amino-acid; the solution absorbed only a trace of bromine in the method of Okuda (11), and a one-fifth aliquot, ignited with the reagent of Denis, yielded less than 1 mg. of BaSO_4 .

TABLE II.
Distribution of Nitrogen in Globin.

	Nitrogen.		Percentage of total nitrogen.			Calculated from analysis of hemoglobin.
	A	B	A	B	Average.	
	mg.	mg.	per cent	per cent	per cent	
Total N taken.....	375.4	457.0				
Ammonia.....	19.3	25.6	5.14	5.60	5.37	5.35
Humic.....	7.0	8.8	1.86	1.93	1.90	1.5
Arginine.....	30.3	36.4	8.07	7.97	8.0	7.85
Histidine.....	47.1	58.6	12.55	12.8	12.7	13.0
Lysine.....	42.4	49.5	11.3	10.8	11.1	11.1
Total bases.....	119.9	144.5	31.9	31.6	31.8	32.0
“ filtrate.....	225.2	277.0	60.0	60.6	60.3	61.2
Amino N of filtrate.....	213.8	259.9	57.0	56.9	57.0	58.2
Non-amino N of filtrate.....	11.4	17.1	3.0	3.7	3.3	3.0
Total N recovered.....	371.1	455.9	99.0	99.8	99.4	(100.0)

Discussion of Nitrogen Distribution.

Considering the somewhat different conditions of hydrolysis under which the two analyses were conducted the agreement between their results is almost surprisingly good. It may be added that two other analyses, carried through upon entirely different preparations of globin, gave figures differing only within the recognized limits of the method from those presented. Our data stand, moreover, in excellent accord with those obtained by Van Slyke (13) in an analysis of ox hemoglobin. This is shown by the figures in the last column of Table II, where Van Slyke's results have been recalculated for globin upon the assumptions that hematin accounts for 2.1 per cent of the total nitrogen of hemoglobin, and that all this appears in the humin fraction.

The proportion of histidine found may be compared with the estimate of Hanke and Koessler (15), whose colorimetric method indicated the presence in horse hemoglobin of 8.8 per cent histidine, corresponding to 13.8 per cent of the total globin nitrogen.

It is perhaps of some interest to attempt from the analytical data an estimate of the number of basic radicles in the globin molecule. This is made possible by the knowledge already gained that 2.12 per cent of the total nitrogen corresponds to four monoamino-acid (tyrosine) radicles. One amino group, therefore, corresponds to 0.53 per cent of the total globin nitrogen. Dividing the percentages of arginine, histidine, and lysine nitrogen by 4, 3, and 2, respectively, and each quotient by 0.53, we find that globin yields 3.8 molecules of arginine, 8.0 of histidine, and 10.4 of lysine; or, taking the integers to which these numbers approximate, 4, 8, and 10 molecules of arginine, histidine, and lysine, respectively. The exact nitrogen percentages to be expected from such a composition (assuming always the precise correctness of our tyrosine determination) are 8.48 for arginine, 12.72 for histidine, and 10.60 for lysine.

A similar calculation based upon the percentage of filtrate amino nitrogen indicates the presence of 108 monoamino-acid radicles in globin.

While such estimates have no claim to precision, they probably approximate pretty closely to the actual composition of the protein. In the case of the bases they can hardly differ by more than unity from the true number.

SUMMARY.

Analytical data are presented which suggest that the globin molecule yields upon hydrolysis 2 molecules of tryptophane, 4 each of tyrosine and arginine, 8 of histidine, 10 of lysine, and approximately 100 other amino-acid molecules, including dicarboxylic acids.

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A STUDY OF SEVERAL CASES OF CYSTINURIA.

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The relation of the cystine elimination to the general catabolism of the other amino-acids in cases of cystinuria has not been satisfactorily studied because of the absence of suitable methods for estimating the amounts of amino-acids and cystine in the urine. The distribution of sulfur, with particular reference to the connection between neutral sulfur and cystine sulfur has not received due consideration for the same reason.

The previous workers in this field, notably Alsberg and Folin,¹ Wolf and Shaffer,² and Williams and Wolf,³ have reached essentially the same conclusions; namely,

1. That in cases of cystinuria there is an increase in the elimination of neutral sulfur and undetermined nitrogen, and a decrease in the excretion of ammonia.

2. That the absolute value of the cystine excreted depends on the amount of protein absorbed and that the ratio of neutral sulfur to total sulfur is relatively greater on low protein intake than on high protein intake.

3. The catabolism of amino-acids other than cystine is not affected.

4. The oxidation of the sulfur contained in cystine is practically complete when the cystine is fed as the free amino-acid and this sulfur is excreted as inorganic sulfate.

5. The sulfur in the food is not absorbed in the form of free cystine, as the cystine in the protein molecule is not utilized unless the protein is previously hydrolyzed.

The conclusions of these authors were based on two assumptions which were not definitely proved. They estimated the cystine from the excess of neutral sulfur remaining after deducting the

¹ Alsberg, C., and Folin O., *Am. J. Physiol.*, 1905, xiv, 54.

² Wolf, C. G. L., and Shaffer, P. A., *J. Biol. Chem.*, 1908, iv, 439.

³ Williams, H. B., and Wolf, C. G. L., *J. Biol. Chem.*, 1909, vi, 337.

amount of neutral sulfur found in the urine of normal individuals. This assumption is true only under certain conditions, as we shall show later. The workers referred to regarded changes in the undetermined nitrogen as an index of variations in the amount of amino-acids in the urine.

In the present paper we make use of several new methods recently published from this laboratory. The cystine is estimated by the colorimetric method of Looney,⁴ the amino-acids are estimated by the method of Folin,⁵ and the distribution of sulfur is determined by the method of Fiske.⁶

The starting point for this investigation was the finding of cystine on the analysis of a renal calculus removed in an operation at the surgical clinic of the Peter Bent Brigham Hospital. The patient, F. T., upon whom the operation was performed serves as the subject for most of the investigation carried out in this study and, therefore, a fairly complete history of this case is given below. Two of the remaining four cases are sisters of the first patient and the other two cases are her sons. These cases were discovered through an analysis of the urines from all the members of the patient's family. The clinical histories follow.

Certain clinical aspects of this case have already been presented in a recent paper by Graves.⁷

F. T. A Russian housewife, age 26 years, entered the hospital complaining of pain in the right side. Her family history was unimportant. A year before admission there had been sudden attack of severe pain in the right upper abdomen, which required morphia for relief. At that time the patient complained of great urgency associated with inability to void more than a few drops of urine at a time. No blood was seen in the urine and x-ray plates gave no evidence of calculi. Physical examinations showed an obese young woman with negative findings, except for slight tenderness in the right upper quadrant. The urine was alkaline, clear, and pale, and contained neither albumin nor sugar. Specific gravity, 1.012. A few white blood cells were found in the sediment. No crystals were observed. X-ray plates again failed to reveal the presence of calculi. A pyelogram on the right side, however, presented in the region of the main collecting portion of the pelvis an area of lessened density. At operation a calculus was removed from the pelvis of the right kidney. This was glistening,

⁴ Looney, J. M., *J. Biol. Chem.*, 1922, liv, 171.

⁵ Folin, O., *J. Biol. Chem.*, 1922, li, 393.

⁶ Fiske, C. H., *J. Biol. Chem.*, 1924, xlvii, 59.

⁷ Graves, R. C., *Ann. Surg.*, 1922, lxxv, 481.

lemon-yellow in color, and on analysis was found to be composed of pure cystine. (Analysis by Dr. Cyrus H. Fiske, Department of Biological Chemistry, Harvard Medical School.)

Sarah (Case 7, Table VII), sister of F. T., weight 180 pounds. Height 4 feet, 10 inches. This patient became obese following her first pregnancy. She feels that the general growth of the child and the development of his hair and teeth have been rather retarded. The patient eats very heartily, especially of meat, fish, potatoes, and bread. Eggs are eaten but seldom. For the past several months she has noted that urination has been somewhat more frequent and that the urine has had a very strong odor. She has also had infrequent pains in the lower abdomen. Microscopic examination of a sterile catheterized specimen of urine shows numerous colon bacilli.

Bertha (Case 8, Table VII), sister of F. T., age 32, weight 200 pounds, height 5 feet, 1 inch. Early health and development were normal. She has had a good deal of trouble with her teeth. Her appetite is good and the diet includes meat once daily. Eggs are eaten very infrequently. There has been no record of any urinary tract.

Barney T. (Case 9, Table VII), age 8, weight 86 pounds. As far as his parents have been able to observe, his physical development has been perfectly normal. There have never been any symptoms of urinary difficulty. He has a voracious appetite and is especially fond of bread and meat. Eggs are eaten with fair frequency.

Maurice T. (Case 5, Table VII, age 6, weight 45 pounds. His growth and development appeared to be normal. His teeth have shown early and rather extreme decay. His appetite is poor and he eats but little meat and eggs.

The last case examined, Mr. S., is the same patient who served as the subject for the investigation of Alsberg and Folin 17 years ago. There has been practically no change in the condition of this patient during the intervening time with respect to his cystine metabolism.

In order to determine the ratio between cystine nitrogen and amino-acid nitrogen in the urine of individuals without any disarrangement in cystine metabolism, eleven samples of such urine were obtained. Seven of these were from normal individuals, the other four were pathological specimens as noted in the table. In each of these samples the cystine content was determined and the cystine nitrogen calculated. The total amino-acid nitrogen was also determined and the value of the ratio cystine nitrogen: amino-acid nitrogen was obtained. The same ratio was found for the urines from three cases of cystinuria. The results of this experiment are given in Table I.

The average amino-acid nitrogen excretion per 100 cc. for the control urines was 8.9 mg. and the average cystine nitrogen was 0.62 mg. The ratio of cystine nitrogen over amino-acid nitrogen was 7.0 per cent.

For the cystinuria cases the figures per 100 cc. of urine were: amino-acid nitrogen 12.3 mg., cystine nitrogen 3.4 mg., and the corresponding ratio was 27.6 per cent. This increase in the ratio

TABLE I.

Urine.	Per 100 cc. urine.			Cystine N Amino-acid N.	Remarks.
	Amino- acid N.	Cys- tine.	Cys- tine N.		
	mg.	mg.	mg.	per cent	
1	10.5	0	0	0	
2	2.55	4.0	0.47	18.4	
3	8.9	8.0	0.93	10.5	
4	9.2	1.5	0.17	1.8	Pleuritis.
5	5.3	2.5	0.29	5.5	
6	9.1	12.0	1.40	15.4	Diabetes.
7	4.8	7.0	0.82	17.1	
8	14.3	10.0	1.2	8.4	Nephritis.
9	11.3	9.0	1.04	9.2	Orthostatic albuminuria.
10	9.2	1.85	0.21	2.3	
11	12.5	3.0	0.35	2.8	
Average....	8.9		0.62	7.0	

Cystinuria.					
I	6.9	13.5	1.57	22.6	
II	9.28	27.0	3.15	34.0	
III	20.0	46.6	5.43	27.1	
Average....	12.3		3.4	27.6	

is due almost entirely to an increase in the numerator (the cystine nitrogen excretion), as can be seen from the table.

This finding is in agreement with the conclusions of the authors quoted and shows definitely that cystinuria patients are able to catabolize the amino-acids with the exception of cystine as well as the normal individual. This conclusion is confirmed by the results recorded in the Tables II to VI, showing the effect of feeding a diet rich in cystine. The subjects came to the hospital fasting and were given a breakfast consisting mainly of eggs.

Tables II, III, and IV give the results obtained when the diet was fed to cystinuric patients and Tables V and VI give the results for normal controls. In Tables II and IV there is a marked increase in the total cystine excretion with a corresponding

TABLE II.

B. T. Age 7 years. Weight 86 lbs.

Time.	Cystine.	Cystine N.	Amino-acid N.	Cystine N Amino-acid N	Remarks.
<i>a. m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
7.35- 9.01	5.8	0.67	7.3	9.2	Breakfast; 3 eggs.
9.01-11.03	15.6	1.82	5.34	34.0	
11.03- 1.10	10.8	1.26	4.86	26.0	
<i>p. m.</i>					
1.10- 2.05	3.9	0.45	4.79	9.4	
2.05- 3.07	6.2	0.72	4.75	15.2	

TABLE III.

M. T. Age 5 years. Weight 45 lbs.

Time.	Cystine.	Cystine N.	Amino-acid N.	Cystine N Amino-acid N	Remarks.
<i>a. m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
7.27- 8.54	1.59	0.185	2.7	6.8	Breakfast; 4 eggs.
8.54-11.05	3.15	0.367	3.3	10.9	
11.05-12.25	2.97	0.346	3.1	11.1	
<i>p. m.</i>					
12.25- 1.09	2.33	0.271	2.9	9.3	
1.09- 2.21	2.80	0.326	3.5	9.3	
2.21- 3.11	2.10	0.245	3.6	6.8	

increase in the cystine nitrogen: amino-acid nitrogen ratio. The case represented in Table III is not quite so clear. Here the excretion of cystine is not markedly increased and this alone would not be sufficient to class the patient as a cystinuric, but there is a definite increase in the cystine nitrogen: amino-acid nitrogen

TABLE IV.

E. T. Age 26 years. Weight 180 lbs.

Time.	Cystine.	Cystine N.	Amino-acid N.	$\frac{\text{Cystine N.}}{\text{Amino-acid N.}}$	Remarks.
<i>a. m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
7.15-8.38	30.3	3.5	15.9	22.0	Blood I. Sample taken at 8.45 a.m. Breakfast; 6 eggs.
8.38-10.00	33.7	3.9	19.0	20.6	
10.00-12.00	55.8	6.5	20.7	31.1	
<i>p. m.</i>					
12.00-2.00	53.8	6.25	22.3	28.0	Blood II. Sample taken at 3.00 p.m.
2.00-4.00	36.6	4.25	18.6	22.8	
4.00-6.00	20.0	2.4	12.8	18.7	

TABLE IV a.

	Amino-acid N.	Urea N.	Non-protein N.
Blood I.			
Whole	7.9	11.0	31.0
Plasma	6.0	11.0	21.0
Blood II.			
Whole	8.1	12.0	32.0
Plasma	6.4	12.0	26.0

TABLE V.

H. B.

Time.	Cystine.	Cystine N.	Amino-acid N.	$\frac{\text{Cystine N.}}{\text{Amino-acid N.}}$	Remarks.
<i>a. m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
6.11-9.18	0.56	0.065	6.45	1.0	Breakfast; 6 eggs.
9.18-11.41	1.84	0.214	8.10	2.6	
11.41-1.02	0.89	0.104	9.50	1.1	
<i>p. m.</i>					
1.02-2.07	0.82	0.095	9.25	1.0	

ratio so that we feel justified in stating that there is a slight disarrangement in the cystine metabolism.

In the two normal controls, Tables V and VI, there is a very slight increase in the cystine output, but the cystine nitrogen: amino-acid nitrogen ratio remains unchanged or slightly decreased. In Table IV, the results for the patient, F. T., show that the excretion of cystine is about ten times as great as in the normal individual.

The changes in the nitrogen distribution of the blood before and after the meal are also given in Table IV *a*. These are found to be within normal limits.

TABLE VI.

M.Y.

Time.	Cystine.	Cystine N.	Amino-acid N.	Cystine N Amino-acid N.	Remarks.
<i>a. m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
7.05- 8.55	3.31	0.386	5.85	6.6	Breakfast; 6 eggs.
8.55-10.50	3.75	0.437	7.80	5.6	
10.50- 1.00	1.54	0.180	6.60	2.7	
<i>p. m.</i>					
1.00- 2.30	3.66	0.426	8.70	4.9	
2.30- 4.15	1.91	0.222	6.60	3.4	

In Table VII we have collected the results of feeding a cystine-rich diet consisting of six eggs on the rate of cystine excretion on six cases of cystinuria and four normal controls.

The urine was collected for a period of 2 hours before feeding the breakfast which consisted essentially of six scrambled eggs. The urine was then collected every 2 hours and analyzed for cystine. The results are expressed in cystine excretion in mg. per hour. After 6 hours the subjects were given a light protein-free lunch.

An analysis of this table will reveal a very marked difference in the behavior of the normal and cystinuric subjects. In the first four cases (normal individuals) there is a moderate increase in the rate of cystine excretion but it never exceeds 4.0 mg. per

hour. The rate for the cystinuric cases is very much higher than this and in Case X reaches a maximum figure of 55.8 mg. per hour. The last five cases show a marked increase in the cystine output following the ingestion of the eggs. This increase reaches a maximum at about the end of the 4th hour and then subsides to the fasting level at the end of the 8th hour. The fasting value for Case VIII is not greatly in excess of that for the normal cases, but the change caused by the diet clearly distinguishes this from the normal cases. Case V has been placed as a border-line case. Here the cystine excretion follows closely that of the normal individuals, but when we examine the ratio of cystine nitrogen: amino-

TABLE VII.
Cystine Excretion per Hour after Taking 6 Eggs.

Case No.....	I	II	III	IV	V	VI	VII	VIII	IX	X
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Before	0.56	3.31	2.71	1.32	1.59	25.7	12.9	3.78	5.8	30.3
Breakfast; 6 eggs.										
2 hrs. later	1.84	3.75	1.98	1.26	3.15	27.3	29.7	4.44	15.6	33.7
4 " "	0.89	1.54	2.28	3.72	2.97	35.3	34.8	13.3	10.8	55.8
6 " "	0.80	3.66	1.85	3.33	2.33	34.1	20.6	9.36	3.9	53.8
Protein-free lunch.										
8 hrs. later	0.82	1.91		3.00	2.30	39.1	14.2	5.28	6.2	36.6
10 " "					2.10					20.0

acid nitrogen as recorded in Table III it would appear that this case also represents a slight degree of cystinuria. In this connection it may be of interest to note that this child is the brother of Case IX and the son of Case X.

In Table VIII we have tabulated the results obtained from feeding 2 gm. of pure cystine from wool to the patient F. T. During the 8 hours immediately following the ingestion of this cystine the patient excreted only 41.7 mg. of cystine in excess of the amount that she was excreting while fasting. The quantity expected if the patient had absorbed the cystine and not utilized it would have been nearly fifty times as much as this. There seems to be no reason to believe, therefore, that pure cystine is

not utilized by the cystinuric individual. This experiment also confirms the findings of Alsberg and Folin.

In Tables IX and X we have tabulated the distribution of sulfur in the urines of one normal control and one cystinuric patient, after the ingestion of six eggs. The cystinuria case chosen was that of Mr. S., the subject investigated by Alsberg

TABLE VIII.

Cystine Excretion of F. T. after Taking 2 Gm. of Cystine.

Time.	Cystine.	Cystine N.	Amino-acid N.	$\frac{\text{Cystine N.}}{\text{Amino-acid N.}}$	Remarks.
<i>a.m.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
7.00-9.00	32.5	3.79	14.4	26.3	2 gm. cystine.
9.00-11.00	45.3	5.27	18.1	29.1	
11.00-1.00	38.9	4.53	16.8	27.0	
<i>p.m.</i>					
1.00-3.00	35.5	4.13	12.0	34.4	
3.00-5.00	52.0	6.07	13.6	44.6	

TABLE IX.

Sulfur Distribution in Normal Urine.

Mr. O.

Hour.	Cystine.	Cystine S.	Total S.	Total $\text{SO}_4\text{S.}$	Neutral S.	Rest S.	$\frac{\text{Cystine S.}}{\text{Neutral S.}}$
	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>
I	1.32	0.35	15.2	12.8	2.4	2.05	14.6
II	1.26	0.34	21.4	14.6	6.8	6.46	5.0
III	3.72	0.99	18.4	16.2	2.2	1.21	45.0
IV	3.33	0.89	26.1	25.9	0.2		
V	3.00	0.80	34.3	31.6	3.8	3.00	16.6

and Folin. The sulfur determinations are expressed in mg. of sulfur excretion per hour.

In the normal control the excretion of neutral sulfur is fairly constant and corresponds very closely to the 24 hour average given in the control cases of Alsberg and Folin. They found a daily output of neutral sulfur of 170 mg., expressed as SO_3 . The value of the neutral sulfur excretion of our control expressed in

the same terms would be 480 mg. The amount of this sulfur which is due to cystine varies from 5 to 45 per cent.

In the cystinuria patient the value of the cystine sulfur follows very closely the amount of neutral sulfur excreted. The total sulfur metabolism is somewhat lower than that given in the previous investigation of this subject, only 1.9 gm. of sulfur as SO_3 per 24 hour period is obtained instead of 2.9 gm. There is also a drop in the neutral sulfur which agrees with this fall in total metabolism, the 24 hour value of the neutral sulfur expressed as SO_3 is 0.600 gm., while Alsberg and Folin obtained 0.820 gm.

The cystine sulfur accounts for about 85 per cent of the neutral sulfur, the ratio varying from 72 to 100 per cent. On calculating the same ratio from the figures given by Alsberg and Folin, we

TABLE X.
Sulfur Distribution in Cystinuria Urine.

Mr. S.

Hour.	Cystine.	Cystine S.	Total S.	Total SO_3S .	Neutral S.	Rest S.	Cystine S Neutral S'
	mg. per hr.	mg. per hr.	mg. per hr.	mg. per hr.	mg. per hr.	mg. per hr.	per cent
I	25.7	6.86	18.9	13.8	5.1	0	100
II	27.3	7.28	23.7	15.4	8.3	1.0	88
III	35.3	9.42	26.9	15.8	11.1	1.7	85
IV	34.1	9.10	36.9	24.3	12.6	3.5	72
V	39.1	10.4	51.6	38.5	13.1	2.7	80

obtain a value of 79 per cent. This figure is derived by assuming that all the neutral sulfur in excess of that given by the normal controls is due to cystine. The normal controls give a value of 0.170 gm. of neutral sulfur, while the subject gives a value of 0.820 gm. The difference 0.650 gm., is taken to be due to cystine. Our value for the total cystine sulfur output on a somewhat lower protein intake is 0.480 gm. This indicates that there has been very little change in the ability of this patient to utilize the cystine of the food.

In order to study the effect of diet and medication on the excretion of cystine, the patient F. T., was studied for a period of 17 days while on a constant low protein intake. The figures for the total nitrogen excretion given in Table XI are sufficient evidence that the protein intake was kept remarkably constant and low.

Time, Feb. to Mar. 1922.	Urine volume.	Cystine.	Cystine N.	Amino-acid N.	Cystine N per cent	Total N. wt.	Amino-acid N per cent	pH.	Acidity (-) cc./100 ml.	Ammonia N. mg.	Remarks.
I	1,610	750 (74)*	86.4	322	26.8	6.90	4.65	7.1	-115	346	Sediment; large amounts of cystine crystals.
II	460	546 (222)	63.6	280	22.7	5.66	4.95	6.3	-186	300	" " "
III	910	484 (93)	56.4	234	24.1	5.54	4.2	6.5	-93	218	" " "
IV	1,150	427 (25)	49.7	242	20.5	5.10	4.7	6.5	-118	212	" " "
V	1,130	431 (2)	50.2	250	20.0	4.57	5.5	8.5	-103	100	15 gm. sodium bicarbonate. Sediment; cystine crystals still present.
VI	955	336	39.1	255	15.3	3.85	6.6	8.6			15 gm. sodium bicarbonate. Sediment; single cystine crystals still present.
VII	1,160	260	31.3	246	12.7	3.52	7.0	8			15 gm. sodium bicarbonate. Sediment; no crystals.
VIII	1,270	180	21.0	266	7.9	3.72	7.15	8.5			15 gm. sodium bicarbonate. Sediment; a few crystals.
IX	1,280	118	13.2	240	5.7	3.36	7.15	8.4			15 gm. sodium bicarbonate. Sediment; a few cystine crystals present.
X	1,145	183	21.3	260	8.2	3.54	7.35	8.8			20 gm. sodium bicarbonate. Sediment; no cystine crystals.
XI	1,275	178	20.8	255	8.1	3.65	7.0	8.6			20 gm. sodium bicarbonate.
XII	1,375	165	19.2	245	7.8	3.22	7.6	8.5			20 " " "
XIII	1,500	270	31.5	225	14.0	3.51	6.4	7.6			Bicarbonate discontinued. Sediment; no crystals.
XIV	1,575	394	45.9	188	24.4	3.76	5.0	6.9			Sediment; no crystals.
XV	1,540	277	32.3	136	23.7	3.51	3.9	6.7			4 gm. atophan. Sediment; no crystals.
XVI	785	338	39.4	197	20.0	3.49	5.65	6.7			Atophan discontinued. Sediment; no crystals.
XVII	920	386	45.0	162	27.7	3.78	4.3	6.7			Sediment; no crystals.

* The figures in parentheses indicate the amount of total cystine present in the form of crystals.

The cystine and amino-acid analyses were made according to the methods given in the first part of this paper and the other analyses according to the methods described in the Laboratory Manual of Biological Chemistry by Folin.

In the determination of the hydrogen ion concentration of the urine, no precaution was taken to prevent loss of CO_2 , as the values were determined merely to show what the effect of bicarbonate was.

The change from a high protein to a low protein diet resulted in a gradual decrease in the amount of cystine eliminated. In 5 days, during which the total nitrogen fell from 6.90 gm. per day to 4.57 gm., the cystine excretion showed a corresponding decrease from 0.750 to 0.431 gm. There was also a marked reduction in the amount of cystine crystals present from a maximum of 0.222 gm. the 2nd day to 0.002 gm., on the 5th. The fall in the total amino-acids was not so marked, the ratio between amino-acid nitrogen and total nitrogen remained practically constant.

Beginning with the 5th day, the patient was placed on an alkali régime, 15 gm. of sodium bicarbonate being given each day in three doses of 5 gm. The alkali was followed by a very marked fall in the amount of cystine excreted. The fall was continuous until the 8th day when a practically constant level was reached. This level was maintained with slight fluctuations until the bicarbonate was discontinued on the 12th day. The amount of cystine excreted returned nearly to the value obtained before the bicarbonate was started within 2 days. During the period of alkali administration there was an increase in the total amount of amino-acids excreted and a marked increase in the ratio between amino-acid nitrogen and total nitrogen.

This experiment would suggest that the use of sodium bicarbonate is of definite value in the treatment of cystinuria. Additional evidence may be found in the progress of the patient whose physical condition very markedly improved under the regimen of low protein diet and alkali medication. The discomfort in the region of the right kidney which had previously been very persistent almost entirely disappeared. It is noteworthy that upon one occasion following the neglect of her diet and the omission of sodium bicarbonate she suffered a recurrent attack of rather severe renal colic which terminated with the passage of two small calculi. It is,

of course, impossible to judge concerning the permanence of the favorable effect of this treatment. It is also true that it is rather difficult to secure the complete and faithful observation of dietary regulations. A final routine prescribed a low protein diet which was supervised by the dietitian of the hospital, and 15 gm. of sodium bicarbonate were ordered to be taken daily for 4 days each week. This seemed to provide sufficient alkali.

These results seem to indicate a far reaching change in the metabolism of the amino-acids which is worthy of further study. No explanation of this difference in behavior of the cystine and the other amino-acids can be offered without considerably more work on the exact method of utilization of amino-acids. The decrease in cystine is not, however, due to a loss while in the urine, as a specimen of urine preserved with toluene gave the same reading for the total color-producing substances after the addition of sodium sulfite even when kept for 2 months. There was a decrease in the amount of color produced directly so that the amount of cystine apparently increased.

The giving of 4 gm. of atophan caused a decrease in the elimination similar to that obtained by bicarbonate. In this case, however, the total amino-acid output was diminished rather than increased. When the atophan was discontinued the values for these substances returned to those obtained before any treatment was given.

The values for the various sulfur determinations are tabulated in Table XII. The total sulfur output per day decreased steadily on the low protein diet and became practically constant on the 6th day. The bicarbonate therapy caused no appreciable change in this value. The total sulfates, both inorganic and ethereal, continued to decrease while the bicarbonate was being taken, and then increased.

The atophan caused a diminution in both the total sulfur and total sulfates.

The neutral sulfur decreased slightly with the change to the low protein intake and remained very constant during the period of alkali therapy. When the bicarbonate was stopped the neutral sulfur again began to drop except for the period when atophan was given.

TABLE XII.

F. T. Cystinuria.

Day.	Urine volume, cc. per 24 hrs.	Total sulfur, mg. per 24 hrs.	Total sulfates, mg. per 24 hrs.	Neutral sulfur, mg. per 24 hrs.	Cystine sulfur, mg. per 24 hrs.	Cystine S, Neutral %	Rest neutral sulfur, mg. per 24 hrs.	Remarks.
I	1,610	104 (124)*	237	187	199	100	0	
II	460	264 (323)	197.5	126	145	100	0	
III	910	292 (317)	185	132	129	98	3	Catamenia.
IV	1,150	329 (335)	179	156	113	73	43	Catamenia.
V	1,130	305	170.5	135	115	85	20	15 gm. sodium bicarbonate.
VI	955	296	136.2	160	89	56	71	15 " "
VII	1,160	309	140.5	169	71	42	98	15 " "
VIII	1,270	292	123.7	168	48	28	120	15 " "
IX	1,280	290	124	166	31	19	135	15 " "
X	1,145	272	106	166	49	29	117	20 gm. sodium bicarbonate.
XI	1,275	278	108.5	170	47	28	123	20 " "
XII	1,375	278	121.5	157	44	28	113	20 " "
XIII	1,500	297	142.5	155	72	56	83	Bicarbonate discontinued.
XIV	1,575	292	187	105	105	100	0	
XV	1,540	227	111	116	74	64	42	4 gm. atophan.
XVI	785	192	75	117	90	77	27	Atophan discontinued.
XVII	920	274	107	97	102	100	0	

* The figures in parentheses give totals when S in cystine crystals has been added.

The sulfur calculated from the cystine figures accounts for all the neutral sulfur on the days on which there was no bicarbonate or atophan taken. The amount of neutral sulfur not accounted for by the cystine, called rest sulfur in the table, gradually increased with the giving of bicarbonate so that the ratio between

TABLE XIII.

Experiment Showing Endogenous Excretion of Cystine.

Fasting for 12 hours since noon; urine collected every 2 hours beginning at midnight.

Time.	Urine. volume.	Cystine.	Remarks.
1921	cc. per hr.	mg. per hr.	
Dec. 22.			
12 n.			Began fast.
Dec. 23.			
12.00-2.00 a.m.	40	2.80	200 cc. water.
2.00-4.00 "	55	1.98	200 " "
4.00-6.00 "	79	3.36	200 " "
6.00-8.00 "	116	2.76	200 " "
8.00-10.00 "	70	2.24	200 " "
10.00-12.00 n.	130	1.56	200 " "
12.00-2.00 p.m.	152	3.04	200 " "
2.00-4.00 "	130	3.12	200 " "
4.00-6.00 "	60	1.44	200 " "
6.00-8.00 "	18	2.70	7 p.m. Vomited 200 cc. very acid gastric juice.
			7.30 p.m. Lunch: 2 buns, glass of milk.
8.00-10.00 "	38	2.85	
10.00-1.45 a.m.	46	2.80	200 cc. water.
Dec. 24.			
1.45-8.45 a.m.	14	2.71	
8.45-10.45 "	22	1.98	6 eggs at 9 a.m.
10.45-12.45 p.m.	38	2.28	
12.45-1.36 "	24	1.85	

cystine sulfur and neutral sulfur dropped from 100 to 28 per cent on the 8th day and remained at this level until the bicarbonate was discontinued. Then the ratio returned in 2 days to its former value of 100 per cent. The atophan also caused a temporary fluctuation in the amount of rest sulfur of a similar nature.

This result is certainly very remarkable. That the bicarbonate alters the metabolism of cystine sulfur is unquestioned, but the manner in which this change is accomplished is completely hidden. The total sulfur excretion is practically constant and there is a fall in the total sulfates eliminated so that it is clear that the cystine sulfur is not completely oxidized. Whether there is a partial oxidation of the sulfur so that it does not appear as cystine or whether there is a combination of the cystine which cannot be determined by the method used can only be surmised. The problem necessitates further investigation.

Table XIII is introduced to show the effect of fasting on the cystine output of a normal individual.

The subject began to fast after a light lunch at noon on December 22, 1921. After 12 hours the urine was collected every 2 hours and the cystine output estimated in mg. per hour. 200 cc. of water were taken every 2 hours. When the fast had continued for 31 hours the subject vomited about 200 cc. of very acid gastric juice. Unfortunately, this material was lost so that no analysis of it could be made.

The table shows that there is a slight, relatively constant excretion of cystine derived from the endogenous metabolism which is not increased by the taking of food rich in cystine.

SUMMARY.

The results recorded in this investigation confirm most of the conclusions of Alsberg and Folin, and of Wolf and his coworkers.

The excretion of cystine is not simply an index of a general disturbance in the metabolism of the amino-acids but a definite entity confined to cystine.

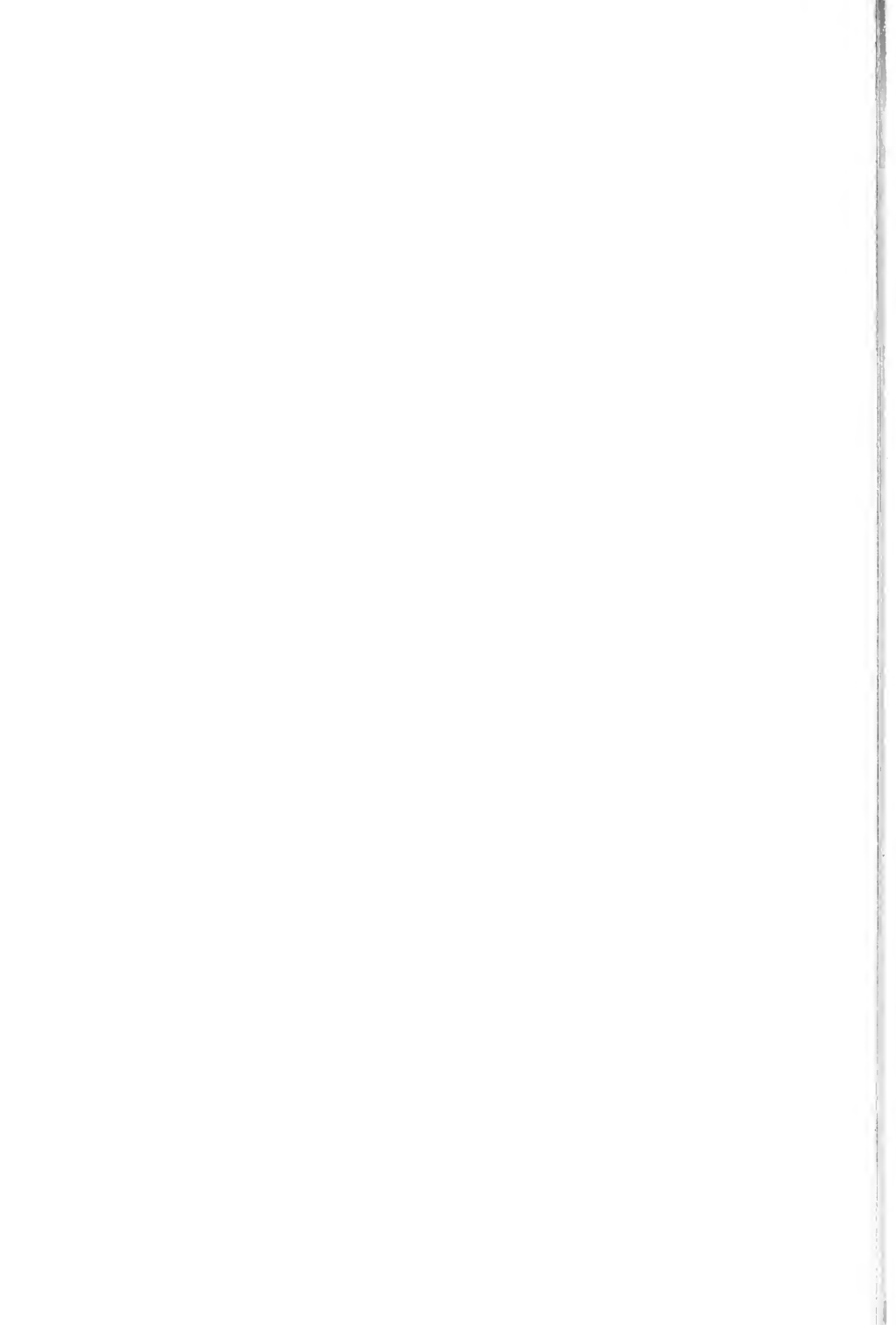
The cystine sulfur accounts for practically all the neutral sulfur found in cystinuria urines provided the patient has not been taking sodium bicarbonate or atophan.

The administration of sodium bicarbonate causes a marked disturbance in the distribution of neutral sulfur due to the decrease in cystine eliminated. This disturbance is not confined solely to the metabolism of cystine as the other amino-acids are also affected, but in the reverse direction. No explanation is attempted for the remarkable changes caused by the bicarbonate, but it is hoped that further work will clear up the situation.

The total amount of cystine excreted depends on two factors: (1) a relatively small but constant amount derived from the endogenous metabolism, and (2), a large fraction which varies in proportion to the protein intake.

When cystine is fed as the isolated amino-acid only a very small amount is excreted unchanged.

In closing there is opportunity for interesting speculation concerning the fact that of the cases of familial cystinuria above presented, only one gave definite record of disease of the urinary tract with the formation of calculi. For this we have no satisfactory explanation. The element of infection seems not to have been an important factor, for F. T., who persistently formed stones, had no signs of associated infection; while Sarah, who gave no story of calculus disease, had heavily infected urine. The subject is surrounded by the same obscurity which cloaks the problem of urinary calculi in general. For the proper furtherance of our knowledge and to make possible more rational therapeutics, we would strongly urge upon clinicians that the stones which they remove be analyzed in every instance. It is evident in the case of F. T., for example, that the diagnosis of the disease depended upon the analysis of the stone.



THE NORMAL VARIATIONS IN PLASMA HYDROGEN ION CONCENTRATION.

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A considerable amount of data has accumulated in regard to disturbance of the acid-base balance in both experimental and pathological conditions. The normal variations in the alkali reserve of blood and plasma have been well established (Peters, Barr, and Rule; and Van Slyke), but comparatively little attention has been paid to the extent of the normal variation in hydrogen ion concentration of the plasma.

This report presents data on the variation of the plasma pH in normal persons, together with parallel observations on the alkali reserve and on oxygen contents and capacities. The data are also utilized to show the relationship between pK_1 (of Hasselbalch's equation) values of plasma and of whole blood.

Through the courtesy of Dr. S. Goldschmidt and Dr. A. Light and their students, and in cooperation with their regular laboratory work on the physiology of the blood in the Department of Physiology in this University, we obtained venous blood from twenty-seven normal medical students. Every sample was taken without stasis from an elbow vein by the same person, Dr. Light, through a sterile dry needle into Pyrex tubes containing mineral oil and neutral powdered potassium oxalate to make 0.3 per cent. The samples were all taken at the beginning of the laboratory period between 10 and 11 a.m. after an hour's lecture and therefore under uniform conditions. They were all taken in April, 1922.

A portion of the blood was centrifuged at once for about three-quarters of an hour in a full, stoppered tube and the plasma removed with the usual precautions. The pH determinations were made by Cullen's colorimetric method. The readings were

TABLE I.

Blood No.	Name.	Determined.						Calculated.			
		Plasma pH at 38°.	Blood CO ₂ content.	Plasma CO ₂ content.	Oxygen.			CO ₂ tension.	Cell volume* O ₂ capacity 0.465.	ΔpK _i (a).†	ΔpK _i (b).‡
					Content.	Capacity.	Saturation.				
		pH	vol. per cent	vol. per cent	vol. per cent	vol. per cent	per cent	mm.	per cent		
1	C.	7.28	54.9		12.9	22.2	62				
2	So.	7.30									
3	A.	7.30	59.6	70.8	8.8	21.3	42	59.1	45.8	0.053	0.043
4	Yo.	7.31	59.8	70.4	8.7	20.5	43	57.4	44.1	0.049	0.039
5	W.	7.33	52.2	59.6	11.2	17.8	63	46.5	38.3	0.034	0.029
6	S.	7.34	59.8	70.0	9.9	18.2	55	53.6	39.2	0.047	0.041
7	On.	7.35									
8	A.	7.35	57.9		12.0	23.0	52				
9	McK.	7.35	60.4	71.5	12.7	20.2	63	53.4	43.4	0.049	0.040
10	McC.	7.36									
11	Ed.	7.36	57.8		7.9	22.6	35				
12	M.	7.36	58.1		7.7	21.0	37				
13	Miss C.	7.36	58.6	66.9	7.5	17.7	43	48.9	38.1	0.034	0.029
14	Miss R.	7.36	53.6	62.5	8.9	18.8	47	45.8	40.4	0.045	0.039
15	P.	7.37	58.8		12.0	19.6	61				
16	Li.	7.37	56.2		12.9	19.0	67				
17	Bi.	7.37	61.2	68.0	7.4	18.3	41	48.5	39.4	0.017	0.015
18	Mar.	7.37	58.7	68.8	12.8	22.4	57	49.2	48.2	0.046	0.034
19	Qu.	7.38									
20	Hu.	7.38	57.9		14.3	25.3	57				
21	Eb.	7.39	54.5		10.9	21.7	50				
22	R.	7.40	52.0		13.2	20.4	65				
23	Mi.	7.40	60.2	70.7	12.1	20.8	58	47.4	44.7	0.048	0.037
24	Ha.	7.40	55.2	66.3	11.5	20.7	56	44.5	44.4	0.058	0.048
25	Q.	7.40	53.2	62.8	9.0	20.8	43	42.1	44.7	0.048	0.039
26	Col.	7.40	60.1	70.8	13.1	21.9	60	47.5	47.1	0.049	0.037
27	Ho.	7.41	59.2	67.7	13.0	21.7	60	44.5	46.7	0.035	0.025

* Average values of Peters, Bulger, and Eisenman, and of Gram and Norgaard.

† Calculated from Hasselbalch's equation using Bohr's coefficient 0.511 for whole blood at 38° (relative solubility coefficient 0.920).

‡ Calculated from cell volume using Bohr's relative solubility coefficient of 0.81 for red cells and 0.975 for plasma.

all made between 20 and 25°C. and corrected to the pH values at 38°C. by the equation $\text{pH}_{38^\circ} = \text{pH}_t + 0.01 (t^\circ - 20^\circ) - 0.22$, where t = temperature of the reading.

The $[\text{CO}_2]$ content determinations were made with Van Slyke's constant volume apparatus. The oxygen content and capacity measurements were made with Van Slyke and Stadie's technique,

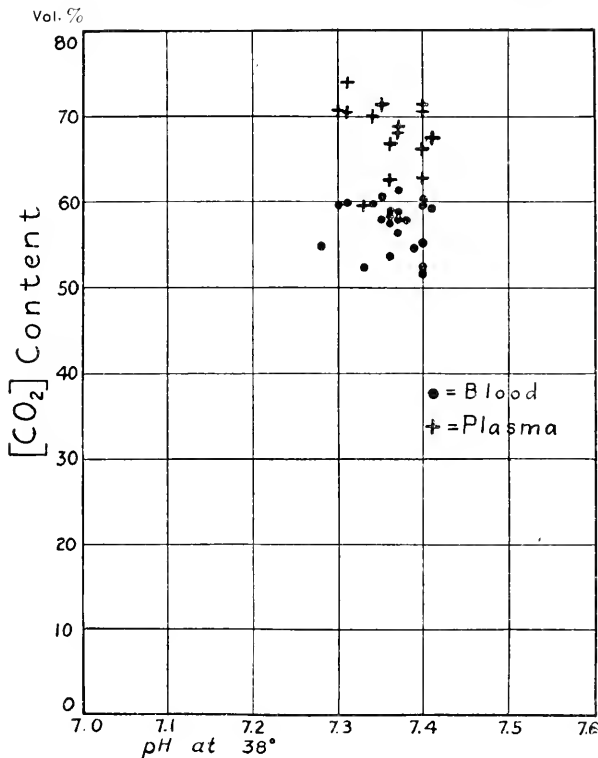


FIG. 1.

using the short form of Van Slyke's apparatus. We are indebted to Dr. J. H. Austin for these oxygen determinations. The whole blood $[\text{CO}_2]$ contents were determined on twenty-three of the samples, and on fourteen of these parallel plasma $[\text{CO}_2]$ content determinations were made.

The results are given in Table I and in Figs. 1, 2, 3, and 4.

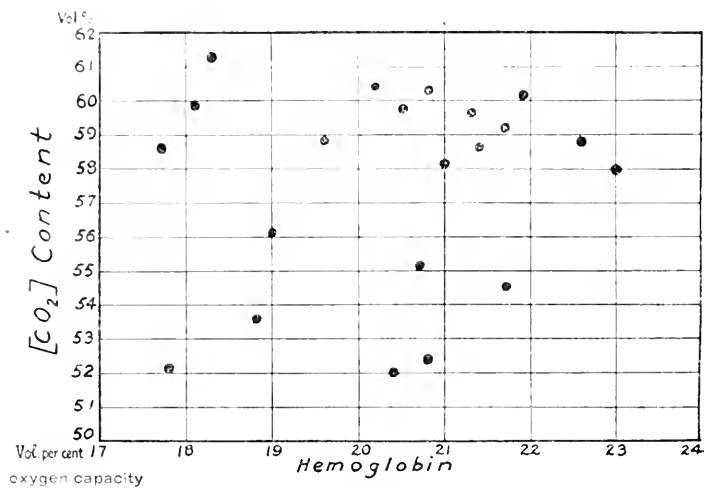


FIG. 2.

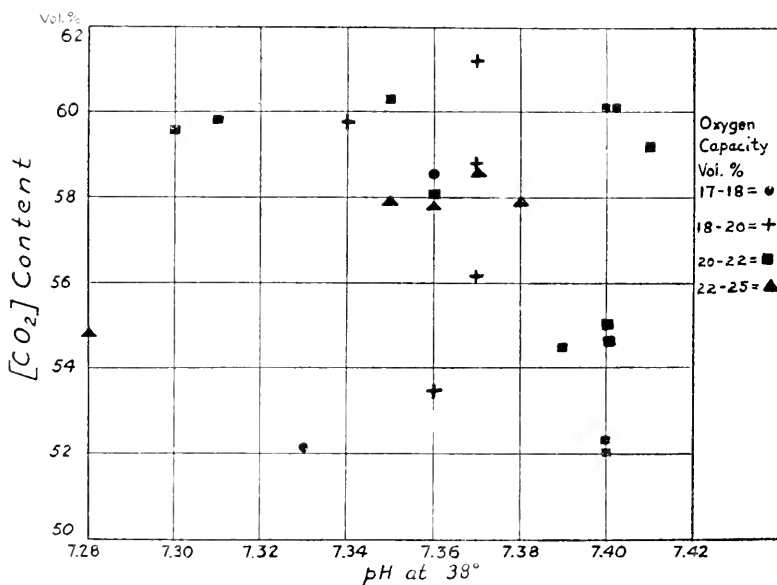


FIG. 3.

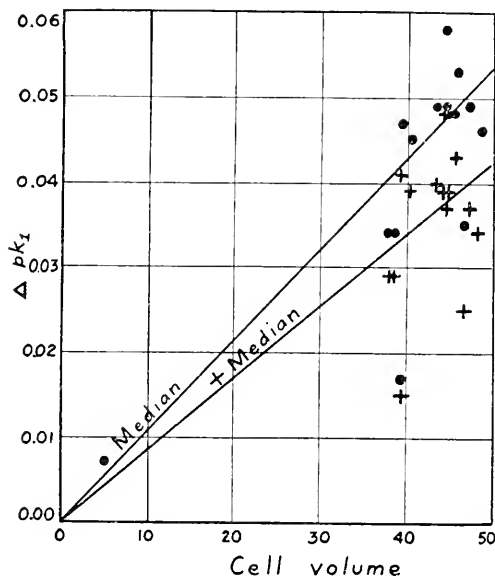


FIG. 4. ● = ΔpK_1 values calculated from Hasselbalch's equation using Bohr's coefficient 0.511 for whole blood at 38° (relative solubility coefficient 0.920). + = ΔpK_1 values calculated from cell volume using Bohr's relative solubility coefficient of 0.81 for red cells and 0.975 for plasma.

Hydrogen Ion Concentration.

The pH of the plasma varied from 7.28 to 7.41. Twenty-one of the twenty-seven determinations lay between 7.35 and 7.40. The maximum variation in pH was 0.13 pH. Even if one assumed that the two extreme values of 7.28 and 7.41 were in error by 0.02 pH (and in the opposite direction) it is evident that any pH values of plasma, measured at 38°, between 7.3 and 7.4 must be accepted as normal.

It is probable that in a similar number of normals taken at random there would be greater variations than in this group where the conditions of age, time of day, activity, occupation, etc., were so uniform.

It is felt that these data lend support to the view that the ordinary normal individual and day by day variations in the reaction of the blood are greater than has been supposed.

Total [CO₂] Content of Blood and Plasma.

In Fig. 1 the total [CO₂] contents of blood and plasma are plotted against pH. Within these normal limits of pH and [CO₂] content there is apparently no systematic relation between the variables, pH and [CO₂] content. In Fig. 2 the whole blood [CO₂] contents are plotted against hemoglobin contents. Again, for this normal range, there is no apparent relation between the level of blood [CO₂] and hemoglobin content.

In order to determine what influence the hemoglobin content of the blood exercised on the position of the blood in the acid-base diagram the blood [CO₂]-pH points of Fig. 1 have been replotted on a larger scale in Fig. 3 and have been divided into four groups according to oxygen capacities—17.7 to 18, 18 to 20, 20 to 22 and 22 to 25 volumes per cent.

It is evident that within this normal range of hemoglobin content any changes in the position of the [CO₂]-pH points on the diagram due to varying hemoglobin are negligible in comparison with the other variations that occur normally.

Difference between Whole Blood and Plasma pK₁.

When both whole blood and true plasma [CO₂] contents are available as well as the plasma pH the data may be utilized to determine the difference between whole blood and plasma pK₁ values, in a manner similar to that used by Peters, Bulger, and Eisenman.

For the purpose of calculation, a plasma pK₁ of 6.10 was assumed¹ and from the pH and [CO₂] content of the plasma, the [H₂CO₃] of the plasma was calculated from Hasselbalch's equation $\text{pH} = \text{pK}_1 + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$. For details of this calculation see the paper on technique (Austin and coworkers). From the plasma [H₂CO₃] using Bohr's solubility coefficient of 0.541 the CO₂ tension at 38° was calculated.

Using this same tension the blood pK₁ values were calculated in two ways. In the first, Bohr's blood solubility coefficient at 38°

¹ The arbitrarily selected value of 6.10 is probably nearly correct. We have recently, on three human plasmas, determined pK₁ electrometrically and obtained 6.081, 6.090, and 6.106.

of 0.511 was used to calculate the blood $[\text{H}_2\text{CO}_3]$ and from this, and the measured value of blood $[\text{CO}_2]$ content, the pK_1 of the blood was obtained. The difference between this and the plasma pK_1 , is given in Column 10 of Table I (ΔpK_1 , *a*)

Warburg, and later, Peters and coworkers have criticized the use of the average value of 0.511 for blood solubility on the ground that the variation in cell volume should be included in the estimation of the CO_2 that is dissolved in whole blood. This contention seems correct and the problem is presented of what value to use for cell CO_2 coefficient. Peters accepts Bohr's values for plasma and cells and uses the equation $(0.7118-0.1205C) \text{pCO}_2 = [\text{H}_2\text{CO}_3]$ where C = the cell volume.

Using this equation the values for ΔpK_1 were calculated and are given in Column 11 of Table I (ΔpK_1 , *b*). The cell volumes were calculated from Peters' value of 0.465 for the oxygen capacity: cell volume ratio which agrees with the values of Gram and Norgaard.

Both the ΔpK_1 values calculated from Bohr's blood solubility coefficient and those calculated from Peters' equation are plotted against cell volume in Fig. 4. Since our pH values are all between 7.30 and 7.40 the influence of pH variations is negligible. It is evident that there exists, over this normal range, a rather large variation in ΔpK_1 values.

It is also evident that the average value of ΔpK_1 of 0.05 calculated by the Bohr solubility factor is about 0.01 higher than the average of the values calculated from Peters' equation. Our results are not of a nature to throw light on the question as to which values are the more accurate. It is worth while, however, to call attention to the difference in the assumptions involved in the two methods. In the first method, the assumption is that Bohr's value of 0.511, established on defibrinated ox blood, is identical with that for normal human blood. The assumption in the equation of Peters, Bulger, and Eisenman—which is derived from the equation $\left(\frac{555}{760} (\text{volume of plasma} \times 0.975 + \text{volume of cells} \times 0.81) = \text{H}_2\text{CO}_3\right)$ —is the validity of Bohr's calculation that the *relative* solubility of CO_2 in red cells, as compared with water, is 0.81. Bohr calculated this from his plasma and whole blood coefficients by assuming a cell volume for his defibrinated ox blood of one-third. The desirability of correcting the whole

blood pK_1 values on the basis of cell volume is unquestioned in the pathological conditions involving changes in cell volume. It seems important also, as Peters and coworkers have done, to establish the normal variation upon the same assumption used in calculating the abnormal. Until the solubility coefficients for normal human blood and cells have been more accurately established, one may arbitrarily choose one or the other of these alternative assumptions. For determining the correction for any given cell volume we may, since $\Delta pK_1 = 0$ when cell volume = 0, draw that line from the origin which passes as a median through the ΔpK_1 value at normal cell volume. In Fig. 4 two such lines are drawn, the upper is the median through the ΔpK_1 values calculated from Bohr's whole blood solubility coefficient, the lower the median through the values calculated from Bohr's plasma and red cell solubility coefficients.

SUMMARY.

Venous blood was obtained under uniform conditions from twenty-seven normal individuals.

On twenty-three of these specimens whole blood $[CO_2]$ content, and oxygen content and capacity were determined, and on sixteen of these true plasma $[CO_2]$ content was also determined.

From these data the difference between the pK_1 of whole blood and plasma has been calculated. The assumptions involved in this calculation are discussed.

pH determinations were made by the colorimetric method on the plasma of these bloods. The pH of the plasma at 38° varied between 7.28 and 7.41. The significance of this normal variation is discussed.

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THE EFFECT OF INSULIN TREATMENT ON THE HYDROGEN ION CONCENTRATION AND ALKALI RESERVE OF THE BLOOD IN DIABETIC ACIDOSIS.

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Reports from other clinics¹ have agreed that in severe diabetes not only the blood sugar concentration but also the alkali reserve is restored to normal under insulin treatment. By a concurrent determination of the $[\text{CO}_2]$ content and the plasma pH it is possible to obtain a more complete picture of the acid-base equilibrium than is afforded by one of these determinations alone. The observations reported here show the behavior not only of the alkali reserve but also of the plasma pH in diabetes during insulin treatment. This study was made in connection with the insulin treatment of diabetes mellitus on the Medical Service of the University Hospital.²

Methods.

Accurate and simple methods are now available for determining the $[\text{CO}_2]$ content of either blood or plasma and the pH of the plasma or serum. We have preferred to use plasma $[\text{CO}_2]$ content rather than blood content, since both pH and CO_2 determinations

* Woodward Fellow in Physiological Chemistry.

¹ Banting, F. G., and Best, C. H., *J. Lab. and Clin. Med.*, 1922, vii, 464. Banting, F. G., Best, C. H., Collip, J. B., MacLeod, J. J. R., and Wake, E. C.; and numerous other reports including the report of Dr. N. B. Foster and R. W. Woodyatt read at the meeting of the Association of American Physicians, May, 1923, and the report of Davies, H. W., Lambie, C. G., Lyon, D. M., Meakins, J., and Robson, W., *Brit. Med. J.*, 1923, i, 847.

² The clinical report of this series is by Dr. L. Jonas, A report of 64 cases of diabetes, in press.

can be carried out on plasma and also because with plasma the influence of changes in the cell volume and oxygen saturation on $[\text{CO}_2]$ content is minimized.

The blood was taken, unless stated otherwise, in the morning before breakfast and before any insulin treatment for that day. This was selected as the best time for obtaining equilibrium conditions. The insulin dosage is given with the dates that indicate changes in dosage.

The blood was drawn into Pyrex tubes containing mineral oil and coated with sufficient *neutral* potassium oxalate to make the concentration 0.3 per cent. The needle, which was dry sterilized, was inserted into the artery at the elbow usually without stasis; when a slight stasis was necessary in order to insert the needle, a half minute was allowed to elapse before the blood was taken. Usually the needle was connected through glass Y-tubes to two tubes, one, for other analyses, was filled first, then the second was filled for acid-base studies. 6 cc. of this blood were removed under oil to a Pyrex tube, stoppered, and centrifuged as previously described (Cullen); the plasma was removed under oil and the $[\text{CO}_2]$ content was determined by Van Slyke's method using the constant volume apparatus. The pH determinations were made by Cullen's colorimetric method; the readings were corrected by the equation $\text{pH}_{38^\circ} = \text{pH}_t + 0.01 (t^\circ - 20^\circ) - 0.22$, where t = temperature of reading. All the pH values are given at 38° . We were assisted in many of these determinations by Mr. H. W. Robinson.

The Folin-Wu method was used for blood sugar determinations.

The CO_2 tensions were calculated from the pH and $[\text{CO}_2]$ content values assuming a pK_1 of plasma of 6.10 and a plasma CO_2 solubility factor of 0.541 (see Austin and coworkers).

Results.

The results are tabulated in Table I and each case is charted in the figures.

In this discussion "acidosis" is used to designate that condition of acid-base balance associated with both decreased alkalinity of blood and decreased alkali reserve; *i.e.*, the "uncompensated alkali deficit" (Area 9) of Van Slyke's diagram.

TABLE I.

Case No.	Date.	Plasma pH at 38°.	Total [CO ₂] con- tent.	CO ₂ tension (calcu- lated).	Blood sugar.	Insulin.	
						Date.	Unit.
	<i>1922</i>	<i>pH</i>	<i>vol. per cent</i>	<i>mm.</i>	<i>gm. per 100 cc.</i>		<i>per day</i>
Ins. 5 M. I.	Nov. 16		17.0		0.411		
	" 18	7.34	41.0	31	0.313	Nov. 16 to 19	32
	" 20	7.50	66.0	36	0.200		
	Nov. 23	7.24	23.0	22	0.174	Nov. 20	24
	" 25	7.48	73.0	41	0.035	Nov. 21 to 22	16
	" 29	7.32	76.0	61	0.094	Nov. 23 to	
	Dec. 1	7.34	70.3	54	0.214	Dec. 5	32
	<i>1923</i>						
	Feb. 23	7.39	60.8	42	0.261		10
Ins. 6* R.	Feb. 12	7.12	17.7	22			20
Ins. 9 B.F.	Feb. 7	7.39	44.4	30	0.333		
	" 13	7.02	15.2	23	0.345	Feb. 8 to 12	20
	" 14	7.39	38.2	27		" 13 to 26	40
	" 15	7.42	59.7	38	0.327		
	" 16	7.43	71.8	42	0.227		
	" 24	7.46	63.3	37	0.188		
	Mar. 2	7.39	58.7	40	0.200	Feb. 26 to Mar. 2	30
Ins. 11 T. C.	Feb. 11	6.98	16.1	26	0.326		
	" 12	7.32	44.0	35	0.306	Feb. 11	30
	" 13						
	a.m.	7.45	59.6	36	0.153	" 12 to 14	40
	p.m.	7.51	64.4	34			
	Feb. 14	7.42	58.7	38			
	" 15	7.43	58.6	37	0.190	Feb. 14 to	30
	" 19	7.41	57.8	38	0.154	Mar. 19	
	" 27	7.47	61.6	36	0.152		
	Mar. 6	7.31	56.0	45	0.228		
	" 10	7.31	47.6	39	0.230		
	" 19	7.22	38.5	38	0.300		
	" 21	7.38	61.4	42	0.190	Mar. 19 to 21	40
	" 22	7.40	67.2	46			

TABLE 1—*Concluded.*

Case No.	Date.	Plasma pH at 38°.	Total [CO ₂] con- tent.	CO- tension (calcu- lated).	Blood sugar.	Insulin.	
						Date.	Unit.
	1923	pH	vol. per cent	mm.	gm. per 100 cc.		per day
Ins. 16 W. R.	Feb. 26	7.31	35.7	29	0.206	Feb. 26 to Mar. 5	30
	" 27	7.40	46.2	30	0.142		
	Mar. 1	7.37	57.2	40	0.234		
	" 3	7.47	74.2	43	0.142		
	" 5	7.50	70.0	38	0.133		
Ins. 22 M. W.	Mar. 13	7.26	38.3	35	0.296	Mar. 13 to 19	30
	" 16	7.40	72.8	49	0.254		
	" 19	7.46	81.5	48	0.284		
Ins. 26 G. B.	Apr. 2	7.27	40.8	36	0.300	Apr. 2 to 5	30
	" 5	7.45	64.3	39	0.314		

*Ins. 6 did not speak English. Unable to get history. Sudden coma, on morning of Feb. 12. Two 10 unit doses of insulin, all that was available, failed to relieve and death occurred at 11 p.m., Feb. 12.

Our results confirm the observations from other clinics regarding the behavior of the alkali reserve under insulin treatment. They show, moreover, that in all the patients with severe acidosis the lowered alkali reserve was associated with a lowered pH. As return toward normal took place under insulin treatment it is striking that the restoration of the pH and the alkali reserve progressed simultaneously. In the figures the slope of the solid lines drawn arbitrarily through the observed points expresses this relationship between the changes in these two factors of the acid-base balance. It will be noted that this slope is similar in the different patients and also in the same patients (Figs. 1 and 2) during two successive periods of acidosis. In one case, Fig. 1, after a normal condition had been attained, there occurred, because of a failure in the insulin supply, a severe acidosis. Later, with sufficient insulin the return of the acid-base equilibrium to normal took place along the same slope as in the preceding recovery. (Compare points 1 and 2 with 3 and 4 in Fig. 1.)

In studying the acidosis of anesthesia Cullen, Austin, Kornblum, and Robinson found that during the recovery from the

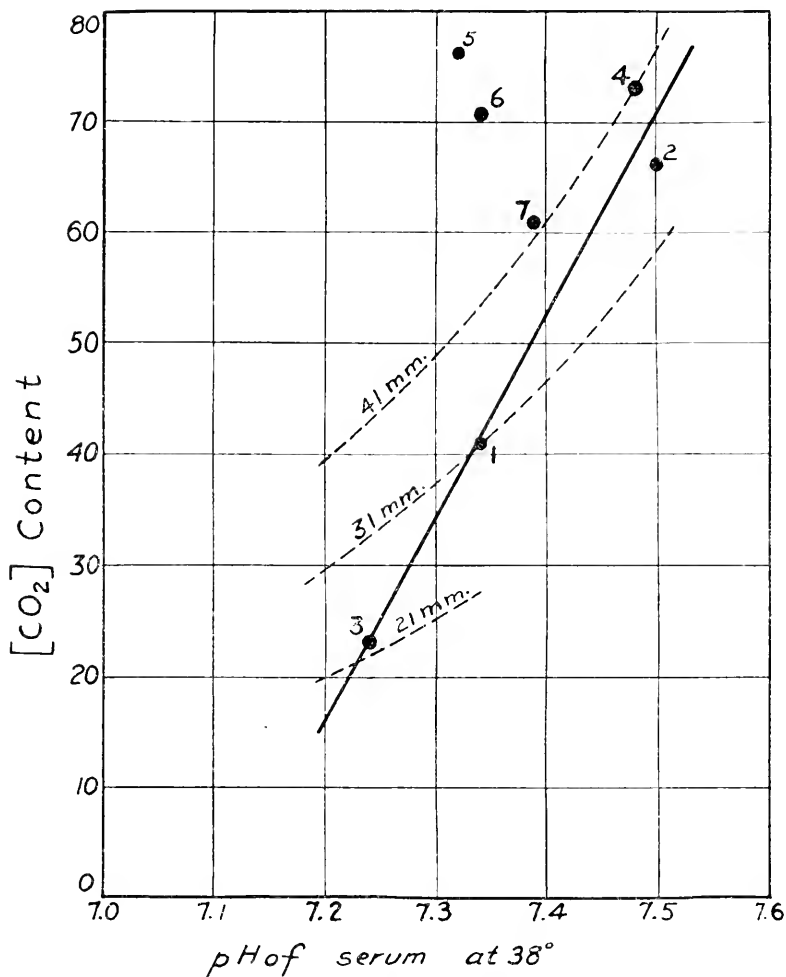


FIG. 1. Insulin 5. M. I., white, female, American, age 34 years, weight 41.3 kilos, admitted Nov. 16, 1922.

The diabetes was of 2 years duration and untreated.

The present crisis followed an acute gastrointestinal disturbance, 3 days before admission.

On admission, the patient was in coma from which she could not be aroused. 500 cc. of a solution containing 5 per cent glucose and 2.5 per cent NaHCO_3 were given by vein. 2 hours later blood was withdrawn for the first examination and 20 units of insulin were given. NaHCO_3 was not given after the above injection.

The patient did well on 32 units a day. On Nov. 20, 1922, because of insufficient insulin, the dose was reduced to 24 units and on Nov. 21 and 22 to 16 units. This caused a relapse from which she recovered on return to 32 units.

The blood ketones expressed in terms of acetone per liter of blood were 1.25 gm. on Nov. 17, 0.18 gm. on Nov. 20, and 0.848 gm. on Nov. 23.

She was discharged Dec. 22, 1922 in good condition and on an insulin dosage of 10 units per day.

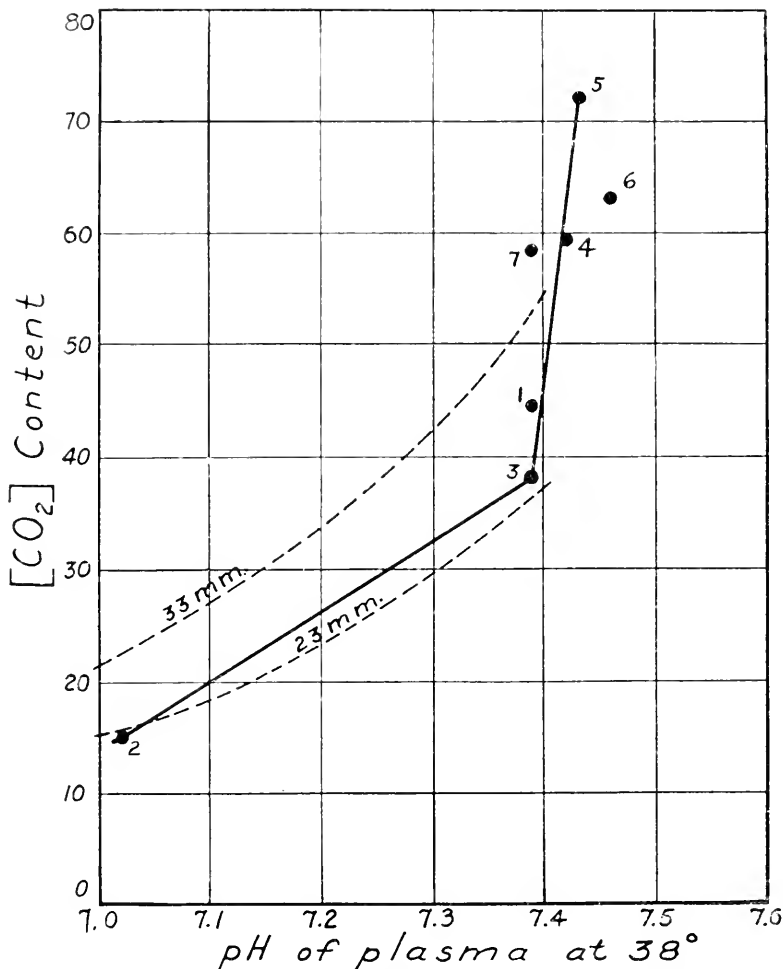


FIG. 2. Insulin 9. B. F., white, female, Russian, age 23 years, weight 37.6 kilos, admitted Jan. 29, 1923.

Diabetes was recognized 6 weeks before admission, at which time she was suffering from numerous furuncles.

On admission, the patient was in a serious condition. She responded slowly to dietetic treatment. On Feb. 28, 1923, insulin treatment was begun, the dosage being 20 units a day. 3 days later a carbuncle developed which greatly intensified the severity of the diabetes. On Feb. 13 respiratory distress and a tendency to stupor developed.

40 units of insulin daily quickly relieved the patient. Insulin was reduced to 20 units and finally stopped because the tolerance improved so that she could take her diet and remain free of ketonuria and glucosuria. She died on Mar. 21 of septicemia.

severe acidosis of anesthesia there was in some, but not all the animals, a suggestion that the recovery proceeded at fairly constant CO_2 tension.

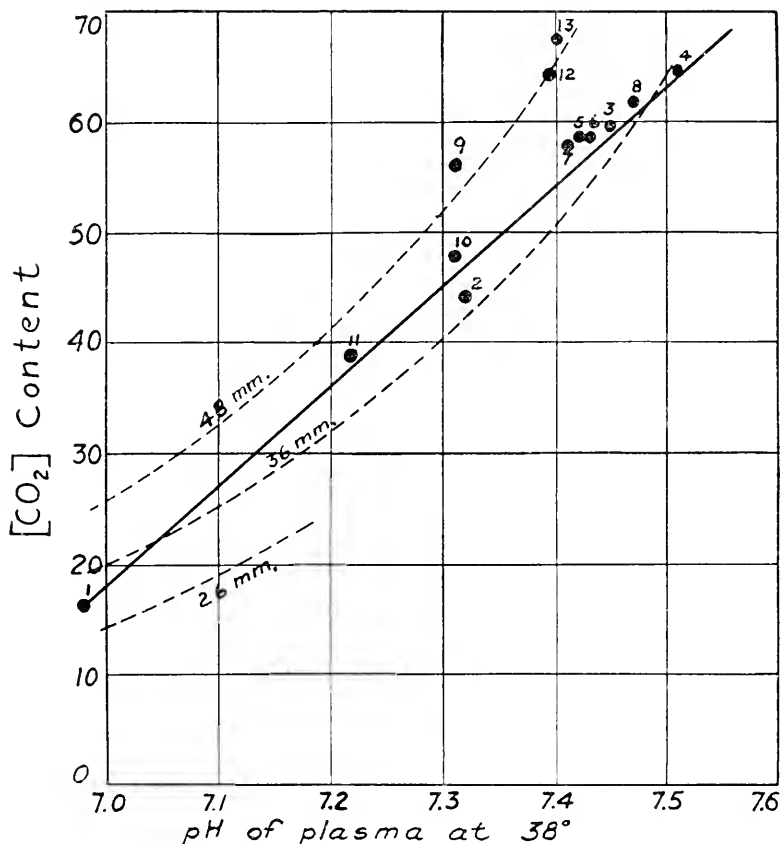


FIG. 3. Insulin 11. T. C., white, male, American, age 18 years, weight 41.5 kilos, admitted Feb. 11, 1923.

The diabetes was of 2 years duration. The present severe condition was caused by acute tonsillitis, 1 week previous to admission.

On admission the patient was semicomatose and showed respiratory distress.

After 2 days of 40 units of insulin a day, 30 units a day were given. On Mar. 19, because of acidosis due to a break in diet, he received 40 units a day for 3 days and thereafter 30 units a day. The blood ketones on Feb. 11 were 0.326 gm. per liter, on Feb. 12, 0.110 gm., and on Feb. 13, 0.074 gm. expressed as acetone.

He was discharged on May 22 free of ketonuria and glucosuria and on a maintenance diet with 30 units of insulin. He weighed 50.5 kilos.

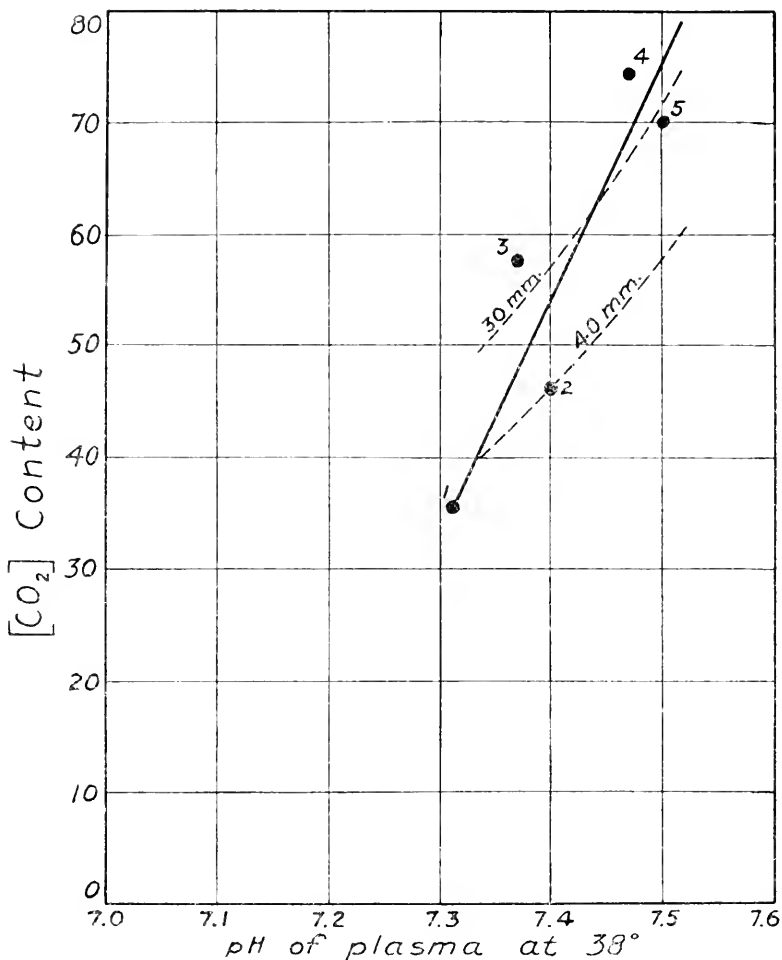


FIG. 4. Insulin 16. W. R., white, male, American, age 35 years, weight 55.8 kilos, admitted Feb. 12, 1923.

The symptoms of diabetes were first noticed 3 weeks before admission. At that time he had an infection of the mouth.

On admission, the symptoms were those of severe diabetes. Failing to respond to dietetic treatment, insulin therapy was begun Feb. 26.

The patient improved rapidly on 30 units of insulin a day.

He was discharged Mar. 21, 1923 in good condition and taking 20 units of insulin per day. He weighed 59 kilos.

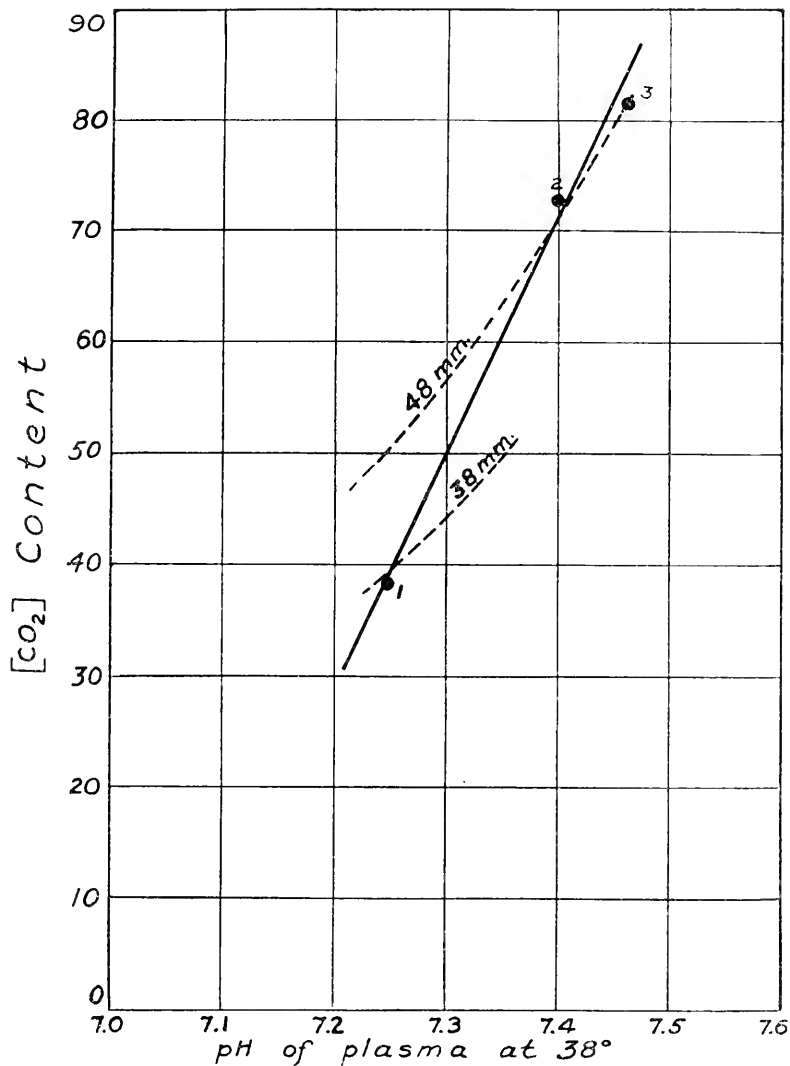


FIG. 5. Insulin 22. M. W., white, male, Hungarian, age 32 years, weight 45.5 kilos, admitted Mar. 12, 1923.

The diabetic history dates back 2 years. A mild diabetic at the onset, with a gradual loss of tolerance until at the time of admission it was impossible to keep him aglucosuric on a maintenance diet.

On admission his symptoms were those of severe diabetes.

The patient responded well to 30 units of insulin a day. On discharge Apr. 21 he was on a maintenance diet with 30 units of insulin daily. He weighed 51.8 kilos.

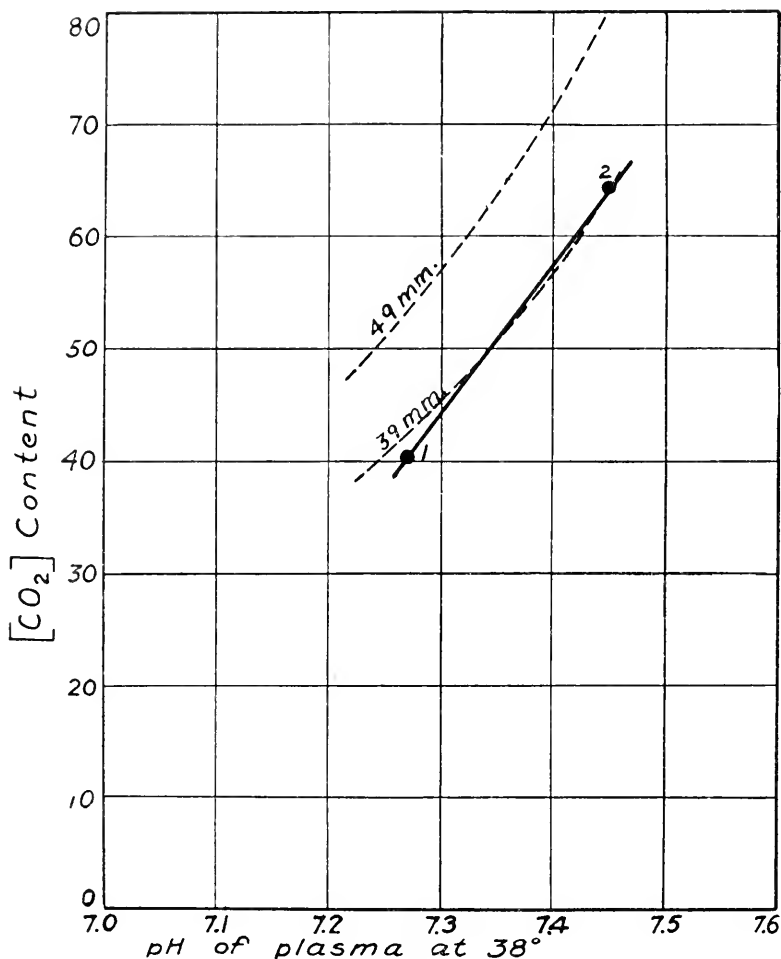


FIG. 6. Insulin 26. G. B., white, male, American, age 26 years, weight 51.5 kilos, admitted Apr. 2, 1923.

The patient had had diabetes for 9 years, mild at first, but with a slowly diminishing carbohydrate tolerance.

The present condition was precipitated by an acute infection of the upper respiratory tract.

On admission the symptoms were those of severe diabetes.

The patient responded well to 30 units of insulin per day. He insisted upon being discharged on the 5th day.

In order to bring out any similar relationship between the CO_2 tension and the changing acid-base balance in these cases, constant CO_2 tension lines (CO_2 isobars) have been drawn as broken curves.

In three of the cases, among them the two with severe acidosis (Figs. 2, 3, and 6), the successive states of acid-base equilibrium have approximated the same CO_2 tension. In one of these cases (Fig. 2) this is true only for the initial stage after which the condition is that of approximately constant pH.

In the remaining three cases reported, the return toward normal was apparently not associated with this constancy of CO_2 tension.

This apparent tendency to maintain constancy of CO_2 tension over extremely wide pH fluctuation may be simply a coincidence, or it may be an indication of a mechanism operating under definite but unknown conditions.

Variations in Reaction.

It is of interest that these observations include the lowest pH yet reported in man with recovery from acidosis; *i.e.*, pH values at 38° of 6.98 and 7.02. That such abnormally low reactions are not in themselves incompatible with recovery is thus demonstrated. Further, after the organism has returned to normal it is apparent that there occurs a considerable fluctuation in the pH. This relatively large day by day fluctuation after recovery is in harmony with the variation in normal pH which we have reported from this laboratory (Cullen and Robinson) and which indicates that the normal variations are larger than have sometimes been considered to be the case. In passing it is interesting to note a case of nephritic acidosis which was studied in this hospital with a pH of 6.7 and a $[\text{CO}_2]$ content of 4 volumes per cent. With strenuous alkali therapy for 36 hours the acid-base balance returned to a pH of 7.25 and a $[\text{CO}_2]$ content of 21.6 volumes per cent and the patient lived for 48 hours.

It would appear that the pH changes which are compatible with life and which indeed occur under conditions of physiological activity are much greater than was formerly supposed. The data reported by Cullen and Robinson for normals, by Barr, Himwich, and Green for conditions of violent exercise, and in this paper for pathological conditions all support this view.

SUMMARY.

The behavior of the acid-base balance has been followed in diabetes during insulin treatment.

The acidosis of severe diabetes is characterized by the lowering of both alkali reserve and plasma pH. Under insulin treatment both alkali reserve and pH return to their normal level coincidently.

There appears to be a consistent relation between these two factors during the return to normal.

Two cases of coma with recovery under insulin treatment with plasma pH at 38° C. of 6.98 and 7.02, respectively, are reported. These are believed to be the lowest values for human plasma pH with recovery that have been reported.

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STROPHANTHIN.

II. THE OXIDATION OF STROPHANTHIDIN.*

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Attempts to obtain information regarding the structure of strophanthidin have been limited to the experiments of Feist¹ and Windaus and Hermanns.² Although the former studied the effect of a number of oxidizing agents, the only definite result obtained by him was by the oxidation of strophanthidin or isostrophanthidin (strophanthidinic acid lactone) with permanganate in alkaline solution after saponification of the lactone group in each of these compounds. In each case, along with oxalic acid and other obscure, amorphous acids, the same crystalline di-basic acid (so called strophanthic acid) was isolated with the melting point 260.8° , and to which Feist attributed the formula $C_{27}H_{35}O_9$. Windaus and Hermanns later confirmed the formation of this acid, but on the basis of analysis, titration, and the analysis of a dimethyl ester adopted the formula $C_{23}H_{30}O_8$, and suggested that its formation might be due to the oxidation of a terminal CH_3 group in the hydroxy-acid, erroneously considered by them to be $C_{23}H_{32}O_6$. From the following, this view will be seen to be untenable.

We have also prepared this acid and from its analysis and titration in the cold, we have confirmed the formula $C_{23}H_{30}O_8$. It is isomeric with the acid to be described later on. However, from its behavior toward alkali, we have found that this acid, contrary to Feist, still possesses a lactone group and is, therefore, a di-basic lactone acid. On acidification the lactone ring readily closes with the quantitative formation of the original

* Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, liv, 253.

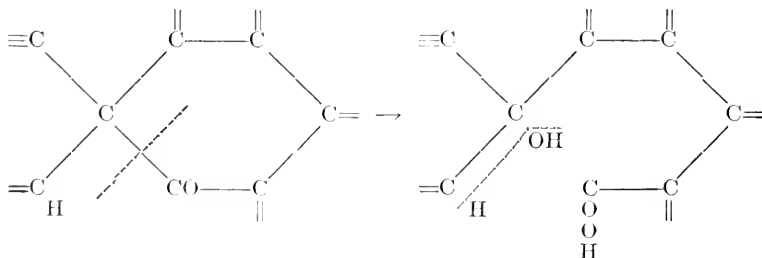
¹ Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2088.

² Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 993.

di-basic acid. Further, the dimethyl ester does not react with ketone reagents, so that the conclusion seems justified that the carbonyl group of strophanthidin is oxidized in the process of the formation of this acid.

The ester forms a benzoate. Accordingly, 7 of the 8 oxygen atoms contained in the acid have been accounted for. This would indicate that the main polycyclic skeleton of the strophanthidin molecule is composed only of carbon atoms. The relationship of this acid to strophanthidin is, at the moment, obscure, but the oxidation of the carbonyl group to carboxyl probably occurs in a manner similar to that discussed in the case of the following substance. Unlike the latter, the lactone ring is probably analogous to that contained in isostrophanthidin.

When strophanthidin is oxidized in the cold in acetone solution with permanganate, a mono-basic acid is obtained, for which the formula $C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$ has been derived. The analysis of the anhydrous substance was rendered difficult by the decomposition experienced when attempts were made to remove the water of crystallization. The above formula was confirmed by analysis of the silver salt and the methyl ester. From its behavior toward boiling alkali, the acid still possesses the lactone ring of strophanthidin. The methyl ester, however, no longer reacts with ketone reagents, and, in this respect, resembles the ester of the previously described di-basic acid. It is suggested, as pictured below, that the carbonyl group is attached to a tertiary carbon atom and, when oxidized, it is changed to carboxyl. The resulting tertiary alcoholic hydroxyl is then removed as water. At the same time 2 hydrogen atoms, elsewhere in the molecule, must be removed. Attempts to detect the presence of olefin linkings by reduction



of the ester with palladium and hydrogen were unsuccessful.

The ester forms a benzoate, so that the alcoholic group is presumably the same as that in strophanthidin which also forms a benzoate. Contrary, however, to the behavior of strophanthidin, which cannot be recovered after saponification of its lactone group, this acid may be, in part, recovered after such treatment.

Attempts to oxidize the above unsaponified acid in neutral solution with permanganate did not yield a tangible product. After saponification, however, this reagent produced a di-basic acid, $C_{23}H_{30}O_8$, isomeric with the previously discussed strophanthic acid of Feist, and Windaus and Hermanns. Contrary to the behavior of the latter, however, the new acid no longer contains the lactone ring. Instead, its dimethyl ester reacts with ketone reagents.

It may be concluded that the secondary alcoholic group contained in the lactone ring of the acid $C_{23}H_{30}O_7$, when once liberated by saponification to the di-basic acid, $C_{23}H_{32}O_8$, is oxidized to the carbonyl group of the acid $C_{23}H_{30}O_8$. Since strophanthidin possesses an analogous lactone ring, the internal ester of this compound is presumably likewise that of a secondary alcoholic hydroxyl group. The dimethyl ester of the acid $C_{23}H_{30}O_8$ still yields a monobenzoate.

Although, on boiling the above acid with alkali, no evidence was obtained of the presence of a lactone group, curiously enough when the alkaline mixture was reacidified, the original acid could not be recovered. Instead, a new di-basic acid was obtained with quite different properties. The analysis of the acid suggested the formula $C_{23}H_{32}O_9$. According to this, its formation was due to the addition of water to the molecule of the original acid. The new acid yielded a dimethyl ester which, in turn, gave an oxime and a monobenzoate. However, a constant error of about 0.6 to 0.7 per cent high in the carbon determinations on the acid, ester, and the benzoate will require explanation.

It is obvious that the oxidative degradation of strophanthidin presents the same difficulties which have been experienced with other members of the group of saturated polycyclic carbon compounds.

EXPERIMENTAL.

The Oxidation of Strophanthidin.

The Acid C₂₃H₃₀O₇.—50 gm. of strophanthidin were dissolved in 2,000 cc. of acetone which had been carefully dried over calcium chloride and then distilled. The solution was turbinated, chilled to 0–5°, and then treated with 25 gm. of powdered potassium permanganate. Oxidation occurred slowly with the gradual deposition of MnO₂ mixed with salts of the acid oxidation products. After about 2 hours during which the temperature was maintained the permanganate was completely used up. The collected precipitate was washed with acetone and then shaken up with water. The filtrate from MnO₂ was concentrated to about 150 cc. and acidified with acetic acid. The crude acid, which partly precipitated as a gum, slowly crystallized on standing and rubbing. The collected substance was suspended in water and dissolved by the addition of ammonia in slight excess. A very small amount of material, which proved to be unchanged strophanthidin, remained undissolved. The gently warmed solution on acidification yielded the acid as colorless rhombic leaflets. The yield was 13 gm.

The acetone filtrate from the above reaction mixture was concentrated to dryness. The resinous residue crystallized when treated with dilute ammonia. The crystals consisted of unchanged strophanthidin (6.7 gm.). The filtrate from the crystals, when concentrated and acidified with acetic acid, yielded a small amount of a gummy precipitate which was followed after seeding by a small quantity of the above described acid. No other crystalline product was obtained from the reaction mixture.

For analysis, the main fraction was recrystallized by dissolving in a small volume of hot 95 per cent alcohol and adding an equal volume of water. The acid slowly deposited as a crust of small, glistening, four-sided, stout plates or leaflets which contained, when air-dry, approximately 0.5 molecule of water of crystallization.

The substance softens above 175° and melts and effervesces at 185–190° although the melting point is considerably influenced by the rate of heating.

$$[\alpha]_D^{20} = +54.8^\circ \text{ (c = 1.005 in methyl alcohol).}$$

It is readily soluble in methyl and ethyl alcohols, acetone, and acetic acid, and very sparingly soluble in chloroform, benzene, and ether. It dissolves completely in carbonate solution. In concentrated sulfuric acid it gives a yellow color which changes rapidly through orange and red to a permanganate purple.

The analysis was made somewhat uncertain by the difficulty of the complete removal of the water from the substance without accompanying decomposition.

Air-dry substance.

$C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$.	Calculated.	C 64.60, H 7.31.
	Found, (a).	" 64.52, " 7.63.
	(b).	" 64.27, " 7.53.

Dried at 100° in vacuo over $CaCl_2$.

$C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$.	Calculated.	H_2O 2.10.
	Found.	" 1.77.

Anhydrous substance.

$C_{23}H_{30}O_7$.	Calculated.	C 65.99, H 7.23.
	Found.	" 65.62, " 7.46.

The acid still possesses the lactone group contained in strophanthidin which was readily determined as follows:

0.1993 gm. of air-dry substance was dissolved in a few cc. of acetone, diluted with water, and titrated with 0.1 N NaOH, using phenolphthalein as an indicator. 4.50 cc. of alkali were required. The calculated volume for $C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$ is 4.67 cc. A solution of 0.2007 gm. of the acid in 25.35 cc. of 0.1 N NaOH was refluxed for 45 minutes and then titrated back. 9.45 cc. of 0.1 N NaOH were used; 9.40 cc. are required by theory.

Strophanthidin, when once saponified by alkali, cannot be recovered as such, but only as the isomeric isostrophanthidin. The above acid, on the contrary, may be partly recovered after saponification. 0.5 gm. was dissolved in 10 cc. of 10 per cent NaOH and allowed to stand 24 hours at room temperature. On acidification with acetic acid, 0.2 gm. of the original acid was recovered as shown by melting point and properties. The mother liquor yielded a small additional amount after concentration.

Silver Salt.—As additional evidence for the formula adopted for the acid, the silver salt was prepared. The acid was dissolved in about 100 parts of water with a slight excess of ammonia which

was then boiled off. The still hot solution when treated with silver nitrate solution yielded glistening six-sided leaflets. The collected salt was suspended in water and dissolved by the careful addition of ammonia. The solution was then diluted and treated with acetic acid in slight excess. On standing, a small deposit of impurities formed from which the clear solution was filtered. After a few days in the refrigerator this deposited a crust of small plates and prisms which were stable to light.

Air-dry substance (dried at 100° in vacuo over H_2SO_4).

$C_{23}H_{29}O_7$ Ag· 2H ₂ O.	Calculated.	H ₂ O 6.42.
	Found.	" 6.65.

Anhydrous substance.

$C_{23}H_{29}O_7$ Ag.	Calculated.	C 52.56, H 5.57, Ag 20.55.
	Found, (a).	" 52.41, " 5.72, " 20.45.
	(b).	" 20.67.

The Methyl Ester.—The above acid was esterified in acetone solution by diazomethane. The ester readily crystallized after removing the solvent. Recrystallized from methyl alcohol, it formed colorless, six-sided tablets which soften above 150° and slowly froth up at about 160–163°. It dissolves in alcohol, chloroform, and hot benzene, and less readily in ether. In concentrated sulfuric acid, the initial yellow color gradually deepens to an orange and finally to an orange-red with a purple fluorescence.

$[\alpha]_D^{27} = 57.6^\circ$ ($c = 1.007$ in methyl alcohol).

Air-dry substance (dried in vacuo at 100° over H_2SO_4).

$C_{24}H_{32}O_7 \cdot H_2O$.	Calculated.	H ₂ O 4.00.
	Found, (a).	" 4.36.
	(b).	" 4.27.

Anhydrous substance.

$C_{24}H_{32}O_7$.	Calculated.	C 66.63, H 7.46.
	Found, (a).	" 66.66, " 8.10.
	(b).	" 66.49, " 7.63.

Attempts to prepare from the ester an oxime and a phenylhydrazone resulted only in the recovery of unchanged starting material. It may be concluded, therefore, that the carbonyl group contained in strophanthidin is no longer present in the acid obtained from it and the free carboxyl group in the latter is presumably formed by oxidation of this carbonyl group.

On the contrary, the formation of a benzoyl compound from the ester would suggest the retention of the alcoholic group of strophanthidin which yields a monobenzoate.

The Benzoate.—1.4 gm. of the methyl ester were dissolved in 20 cc. of dry pyridine and the solution after chilling was treated with 2 cc. of benzoyl chloride. After 1 hour, the mixture was poured into dilute sulfuric acid. The precipitated oil crystallized readily when treated with alcohol. After several recrystallizations from methyl alcohol, it formed colorless minute prisms which melted at 243–244° (corrected). The substance dissolves easily in acetone and chloroform but is practically insoluble in benzene or ether.

$$\begin{aligned} [\alpha]_D^{20} &= 61.0^\circ \text{ (} c = 1.004 \text{ in acetone).} \\ \text{C}_{31}\text{H}_{36}\text{O}_8. \quad &\text{Calculated. C 69.37, H 6.77.} \\ &\text{Found, (a). " 69.10, " 6.76.} \\ &\text{(b). " 69.12, " 6.81.} \end{aligned}$$

Oxidation of the Acid $\text{C}_{23}\text{H}_{30}\text{O}_7$.

The Acid $\text{C}_{23}\text{H}_{30}\text{O}_8$.—Attempts to oxidize further the acid $\text{C}_{23}\text{H}_{30}\text{O}_7$ under conditions in which the lactone group had been left intact have thus far given unpromising results. After saponification, however, the liberated secondary alcoholic group was readily oxidized by permanganate to a carbonyl group.

10 gm. of the acid $\text{C}_{23}\text{H}_{30}\text{O}_7 \cdot \frac{1}{2}\text{H}_2\text{O}$ were dissolved in 100 cc. of 4 per cent NaOH. The solution was allowed to stand at 25° for 1 hour for saponification and was then diluted to 1,000 cc. 200 cc. of 5 per cent permanganate were added to the turbid mixture. The reaction rapidly completed itself, and the temperature rose to 27°. 100 cc. of normal acetic acid were then added and the mixture was filtered. The filtrate was concentrated to about 100 cc. under reduced pressure and then acidified strongly to Congo red with sulfuric acid. A partly crystalline precipitate slowly formed. After standing in the refrigerator for a day the collected acid was dissolved in a necessarily large volume of hot alcohol. Since the substance separated but incompletely on cooling, the filtrate was concentrated to about 100 cc. About 5 gm. of colorless, minute, flat needles or platelets separated. This material even after repeated recrystallization from alcohol gave analytical results which were 0.8 per cent high in carbon.

This was unquestionably caused by a sparingly soluble by-product of the reaction. The acid was eventually purified over the barium salt which will be described below. The barium salt, suspended in a small volume of water, dissolved when acidified with hydrochloric acid. On rubbing, the acid separated. The collected material was dissolved in dilute ammonia and again made to crystallize by addition of acid. It formed, when allowed to crystallize undisturbed, minute, glistening, pyramided prisms which contained 2 molecules of water of crystallization. If allowed to crystallize rapidly, the acid may separate as globular aggregates of minute needles which are practically anhydrous. It crystallizes from alcohol in anhydrous form which melts and effervesces at $276-278^{\circ}$ with preliminary sintering. The hydrate effervesces at $268-270^{\circ}$ with preliminary sintering and discoloration. The latter dissolves fairly readily in hot water, but the anhydrous acid gradually separates. Otherwise, either form of the acid is difficultly soluble in the usual solvents. It dissolves in sulfuric acid with a yellow color which changes through orange to red with a green fluorescence.

$$[\alpha]_D^{25} = 28.0^{\circ} (c = 0.995 \text{ in pyridine}).$$

Air-dry substance (dried in vacuo over H_2SO_4).

$$C_{23}H_{30}O_8 \cdot 2H_2O. \quad \text{Calculated. } H_2O \ 7.66. \\ \text{Found.} \quad \quad \quad \text{" } 8.17.$$

Anhydrous substance.

$$C_{23}H_{30}O_8. \quad \text{Calculated. } C \ 63.56, H \ 6.96. \\ \text{Found, (a).} \quad \text{" } 63.69, \text{ " } 6.94. \\ \text{(b).} \quad \text{" } 63.71, \text{ " } 6.94.$$

0.1644 gm. of anhydrous substance was suspended in a small volume of water and titrated with $0.1 \times NaOH$, using phenolphthalein as indicator. 7.9 cc. were required. Calculated for the molecular weight 434.24, 7.6 cc.

0.0571 gm. of anhydrous substance was refluxed for 1 hour in 15.0 cc. of $0.1 \times NaOH$. 3.00 cc. were required. Calculated for two equivalents, 2.65 cc.

Although the titration after boiling with alkali seems to exclude the presence of a lactone group, the acid cannot be recovered as such but is converted into the acid below.

Barium Salt.—1.5 gm. of the above acid were dissolved in 150 cc. of water containing a slight excess of ammonia and the solu-

tion was treated with barium chloride solution. On concentrating under reduced pressure, the salt separated incompletely as glistening, silky needles. The mother liquor yielded additional amounts. The salt was recrystallized by dissolving in hot water and since it showed little tendency to separate, the solution was concentrated to crystallization. The air-dried salt contained 6 molecules of water of crystallization. For the combustion it was necessary to use lead chromate.

Air-dried substance (dried in vacuo at 100° over H₂SO₄).

C₂₃H₂₅O₈Ba·6H₂O. Calculated. H₂O 15.95.

Found. " 16.30.

Anhydrous substance.

C₂₃H₂₅O₈ Ba. Calculated. C 48.46, H 4.92, Ba 24.12.

Found. " 48.26, " 5.16, " 24.77.

The Dimethyl Ester.—A suspension of the acid in acetone reacted at once with diazomethane. Evaporation of the solvent left a residue of rhombic platelets which were collected with ether. Recrystallized from methyl alcohol, the ester formed four-sided platelets and prisms which sintered at about 246° and melted with effervescence at 251–252°. The ester is soluble in alcohol, chloroform, and acetone, and very sparingly soluble in benzene or ether. It dissolves in concentrated sulfuric acid with a play of color through orange to deep red with a greenish fluorescence.

$[\alpha]_D^{25} = -12.0^\circ$ ($c = 1.000$ in acetone).

C₂₅H₃₄O₈. Calculated. C 64.90, H 7.41.

Found, (a). " 64.84, " 7.27.

(b). " 64.79, " 7.15.

The Benzoate of the Dimethyl Ester.—This was prepared in the usual way from 1.5 gm. of the ester in pyridine solution with an excess of benzoyl chloride. After 1 hour, the reaction mixture when poured into acid gave a slowly crystallizing oil. The collected material was recrystallized from methyl alcohol, the operation requiring a large volume. 1.3 gm. of thin platelets, which melted with decomposition at 249–251°, were obtained. The substance dissolves readily in chloroform and pyridine, appreciably in acetone, and sparingly in hot alcohol. It is practically insoluble in ether and ligroin. In concentrated sulfuric acid it gives the same reaction as the original ester.

$$[\alpha]_D^{20} = 7.5^\circ \text{ (} c = 1.064 \text{ in acetone).}$$

$C_{22}H_{33}O_9$. Calculated. C 67.81, H 6.76.

Found, (a). " 67.73, " 6.61.

(b). " 67.96, " 6.57.

The Phenylhydrazone of the Dimethyl Ester.—1.7 gm. of the ester were heated on the water bath with 20 cc. of acetic acid and 2 gm. of phenylhydrazone. Stout crystals separated after 30 minutes. The collected substance was washed with acetic acid. Recrystallized from methyl alcohol, it formed lustrous platelets with a slightly yellowish tinge which melted and decomposed at $265\text{--}266^\circ$. It is sparingly soluble in the cold in the usual solvents.

$C_{31}H_{40}O_7N_2$. Calculated. C 67.35, H 7.30.

Found, (a). " 67.58, " 7.27.

(b). " 67.75, " 7.27.

The Oxime of the Methyl Ester.—1 gm. of the ester was refluxed for 2 hours in 20 cc. of methyl alcohol together with 0.5 gm. of hydroxylamine hydrochloride and 2 gm. of sodium acetate. The alcohol was boiled off and the residue made to crystallize by the addition of water. From dilute methyl alcohol lustrous, flat needles were obtained which are appreciably soluble in alcohol, acetone, and chloroform and but little soluble in ether or benzene. It melts with decomposition at $272\text{--}274^\circ$ with preliminary darkening and sintering.

$C_{23}H_{35}O_5N$. Calculated. C 62.85, H 7.39.

Found, (a). " 63.00, " 7.48.

(b). " 62.97, " 7.40.

The Acid $C_{23}H_{32}O_9$.—9.5 gm. of the above described acid, $C_{23}H_{30}O_9$, were dissolved in 100 cc. of 2 per cent sodium hydroxide solution and the mixture was then heated on the water bath for 30 minutes. When acidified with 25 per cent sulfuric acid, the original substance was not obtained but a new acid separated on rubbing as a thick paste of delicate needles. The collected acid was washed with ice water. The yield was 7.6 gm. Recrystallized from a small volume of water, the substance separates as colorless, glistening needles, containing when air-dry, 2.5 molecules of water of crystallization. It melts and effervesces at

185–187°. It dissolves easily in alcohol and appreciably in water and acetone. In sulfuric acid it gives at first a yellow color which changes to a red with green fluorescence.

Air-dry substance.

$$[\alpha]_D^{25} = -37.0^\circ \text{ (} c = 1.206 \text{ in pyridine).}$$

Air-dry substance (dried at 100° in vacuo over H₂SO₄).

C₂₃H₃₂O₉ · 2½H₂O. Calculated. H₂O 9.05.

Found. " 8.76.

Anhydrous substance.

C₂₃H₃₂O₉. Calculated. C 61.03, H 7.13.

Found, (a). " 61.63, " 7.02.

(b). " 61.75, " 7.15.

After recrystallization from water, the analytical figures remained unchanged. The high carbon values for this acid as well as the similar high carbon values which were obtained on analysis of the ester to be described below and the benzoate obtained from it are at present difficult to explain. The discrepancy is not great, but it has appeared too consistently to be ignored. The analysis of the oxime, however, has given figures in better agreement with the postulated formula. For the present, we see no reason against the provisional acceptance of the formula C₂₃H₃₂O₉ for the acid. According to this view, it is formed by the addition of a molecule of water to the acid C₂₃H₃₀O₈. That this is not due to the opening up of a lactone was shown above by the titration of the latter acid before and after boiling with excess alkali. In accordance with this, the acid C₂₃H₃₂O₉ when titrated gave figures for a di-basic acid. 0.0890 gm., when suspended in water, required 4.30 cc. of 0.1 N NaOH, using phenolphthalein as an indicator. The calculated volume is 3.95 cc. for a di-basic acid C₂₃H₃₂O₉.

Until further data are obtained, it will be difficult to interpret the relationship of this acid to that from which it was obtained.

The Dimethyl Ester.—A suspension of the acid in acetone reacted smoothly with diazomethane with the formation of a clear solution. After removing the acetone, the ester, which slowly crystallized, was collected with ether. The addition of water to the solution in methyl alcohol caused the gradual deposition of a crust of glistening prisms. Recrystallized again for analysis, the ester melted at 205–206°. Repeated recrystal-

lization as above or from dry acetone did not alter the analytical result, the carbon, as in the case of the acid, being about 0.6 per cent too high. The substance is soluble in alcohol, chloroform, and acetone, and but sparingly so in benzene and ether. In sulfuric acid it gives the same play of colors shown by the acid. In acetone solution, it showed no appreciable rotation, $c = 1.000$.

$C_{25}H_{36}O_9$. Calculated. C 62.46, H 7.52.

Found, (a). " 63.06, " 7.72.

(b). " 63.06, " 7.69.

The Benzoate of the Dimethyl Ester.—1.5 gm. of the ester were benzoylated in 20 cc. of pyridine with 3 cc. of benzoyl chloride. After standing 1 hour the mixture was poured into an excess of dilute sulfuric acid. The pasty precipitate, which could not be made to crystallize, was shaken out with ether. The ether extract, washed successively with acid, water, dilute carbonate solution, and finally water, was dried and concentrated. The colorless syrup slowly, but only partly, crystallized on standing, which was facilitated by the addition of ether. 0.6 gm. was obtained. Recrystallized by dissolving in a small volume of methyl alcohol and adding an equal volume of water, it formed microscopic plates and prisms which melted at $172-174^\circ$. The substance is readily soluble in alcohol, acetone, chloroform, and benzene, and with difficulty in ether.

$C_{22}H_{40}O_{10}$. Calculated. C 65.72, H 6.90.

Found, (a). " 66.33, " 6.94.

(b). " 66.30, " 6.99.

The Oxime of the Dimethyl Ester.—A mixture of 1.5 gm. of the ester, 0.5 gm. of hydroxylamine hydrochloride, and 2 gm. of sodium acetate in 20 cc. of methyl alcohol was refluxed for 2 hours. Concentration of the solution and addition of water yielded a gum which crystallized after a day or so. Recrystallized by addition of water to its methyl alcoholic solution, and seeding, it forms minute, rhombic prisms which when air-dry contain 1 molecule of water of crystallization. This is held very tenaciously and it required rather long heating under reduced pressure over sulfuric acid to remove the water completely for analysis. The substance melts and slowly froths up at $158-160^\circ$.

with preliminary sintering. It is soluble in alcohol and acetone, and very sparingly in chloroform, benzene, and ether.

Air-dry substance (dried at 100° in vacuo over H_2SO_4).

$C_{25}H_{37}O_9N \cdot H_2O$. Calculated. H_2O 3.51.

Found. " 3.30.

Anhydrous substance.

$C_{25}H_{37}O_9N$. Calculated. C 60.57, H 7.53.

Found, (a). " 60.38, " 7.69.

(b). " 60.36, " 7.49.

Oxidation of Strophanthidin after Saponification.

Strophanthic Acid.—In all attempts to oxidize strophanthidin by the method of Feist, *i.e.*, permanganate in alkaline solution, we have obtained an obviously impure amorphous acid as the main product of the reaction. The crystalline strophanthic acid of Feist was isolated only in relatively small amount. Since strophanthidin is easily subjected to a rather obscure alteration on boiling with alkali we have attempted to avoid this as much as possible by opening up the lactone group by the method used by Windaus and Hermanns in their titration of this substance. After many experiments the following method was found to give the best results:

50 gm. of strophanthidin were refluxed for 1 hour in a mixture of 5,000 cc. of alcohol and 3,750 cc. of 0.1 N sodium hydroxide solution. The solution was then concentrated under diminished pressure to remove all alcohol, diluted to 5 liters, and then oxidized by the gradual addition of 900 cc. of 5 per cent permanganate solution to the turbid mixture. Toward the end, the permanganate disappeared very slowly. The filtrate was acidified with acetic acid and concentrated under diminished pressure to about 200 cc. The further addition of acetic acid in excess was followed after seeding by the slow deposition of the crystalline acid which was accelerated by warming the mixture. The collected acid was washed with a little 50 per cent acetic acid and then with water. The yield was 7 gm. Acidification of the mother liquor to Congo red with sulfuric acid caused a gummy precipitate to form which could not be made to crystallize. The crystalline acid was suspended in a small volume of 50 per cent alcohol, dissolved by the addition of ammonia, and then repre-

cipitated with dilute sulfuric acid. Recrystallized again from dilute alcohol, it formed needles which melt and effervesce at about 270° (260.8° according to Feist). To the properties recorded by Feist we may add that in concentrated sulfuric acid it gives a yellow color changing gradually through orange to red. In methyl alcohol $[\alpha]_D^{25}$ is -22.0° ($c = 1.000$).

$C_{23}H_{30}O_8$.	Calculated.	C 63.56,	H 6.96.
	Found, (a).	" 63.43,	" 7.33.
	(b).	" 63.45,	" 7.30.

From the analytical data we have adopted, in agreement with Windaus and Hermanns, the formula $C_{23}H_{30}O_8$ for the acid. Contrary to Feist, however, the behavior of the substance towards alkali has shown it to be a lactone acid. The explanation, postulated by Windaus and Hermanns, that the substance originates by the conversion of a CH_3 group to $COOH$ in the acid obtained by saponification of strophanthidin is therefore untenable.

0.2000 gm. was suspended in water and dissolved in the cold by the addition of a slight excess of $0.1\ N$ NaOH. On titrating back to phenolphthalein, 9.10 cc. were used. The calculated amount for a di-basic $C_{23}H_{30}O_8$ acid is 9.18 cc. The opening of the lactone group requires somewhat more vigorous treatment than in the method used by Windaus and Hermanns for strophanthidin. 0.2004 gm. of the acid refluxed for 2 hours in 25.0 cc. of $0.1\ N$ NaOH consumed 14.00 cc. of the alkali. The calculated amount for 3 $COOH$ is 13.80 cc. On acidification of the mixture, the original acid rapidly crystallized and was almost quantitatively recovered. This is contrary to the behavior of strophanthidin which is converted, under these conditions, into an isomeric modification, isostrophanthidin. We believe that this acid is in reality an oxidation product of the latter. Since the ester no longer reacts with ketone reagents, the carbonyl group is no longer present. However, the formation of a benzoate shows the retention of an alcoholic group. In this acid four of the eight oxygens present are accounted for in the two carboxyls, two in the lactone group, and one in an alcoholic group.

The Dimethyl Ester.—This was prepared with diazomethane in accordance with Windaus and Hermanns. These workers

report a melting point of 214° . Our substance melted at $251-253^{\circ}$. The substance was soluble in alcohol, acetone, and chloroform, and with difficulty in benzene and ether. In experiments to prepare the phenylhydrazone the ester was recovered unchanged.

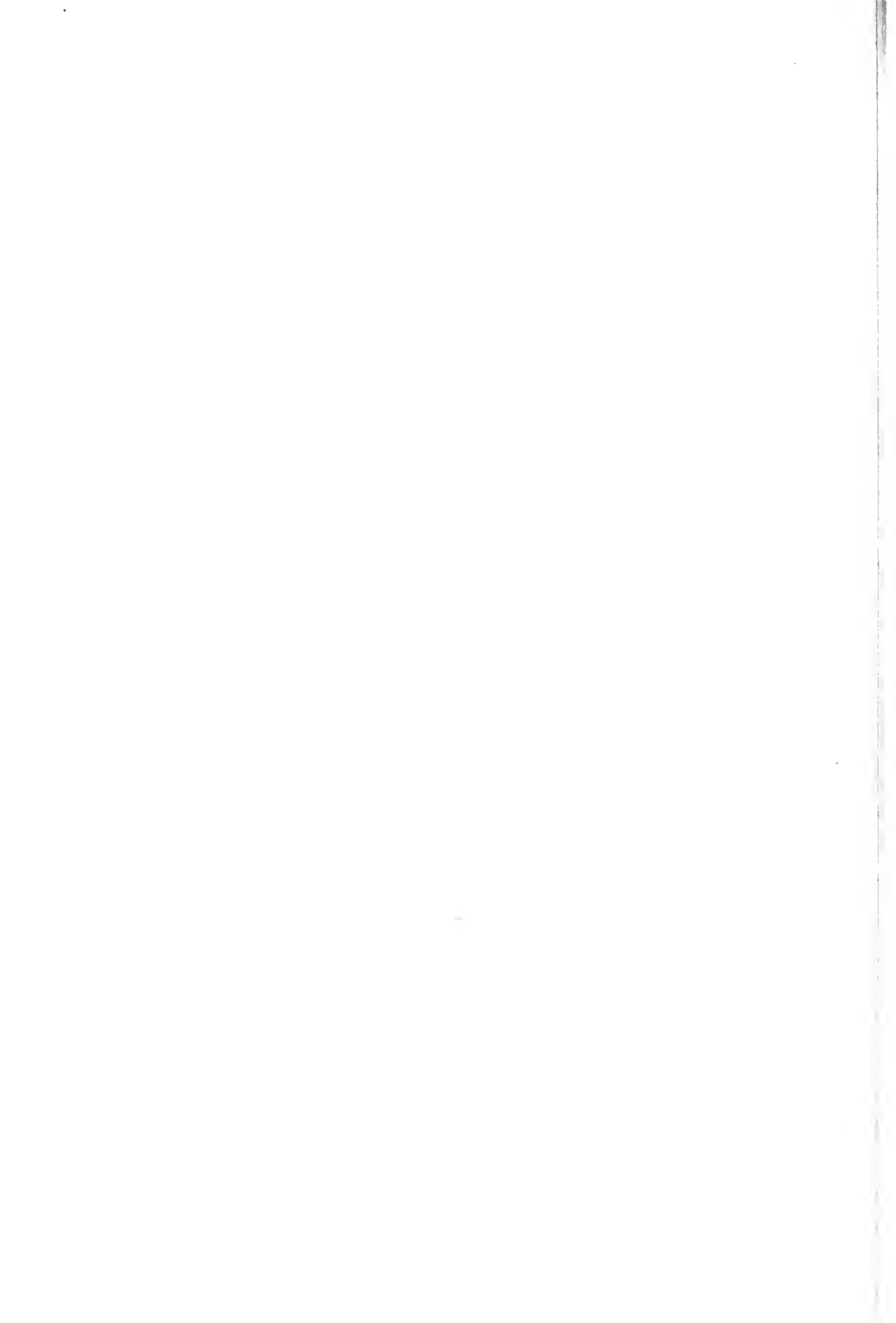
$$[\alpha]_D^{20} = -28.0^{\circ} (c = 0.995 \text{ in methyl alcohol}).$$

$C_{25}H_{33}O_3$. Calculated. C 64.90, H 7.41.
Found, (a). " 64.90, " 7.63.
(b). " 64.70, " 7.51.

The Benzoate of the Dimethyl Ester.—This was prepared as usual from the ester in pyridine with benzoyl chloride. Recrystallized from methyl alcohol, it formed glistening prisms which melt at $233-235^{\circ}$. It is soluble in chloroform and acetone, and particularly on warming in alcohol, ether, or benzene. The solution in acetone quickly deposits delicate needles which probably contain solvent.

$$[\alpha]_D^{20} = -7.0^{\circ} (c = 1.007 \text{ in acetone}).$$

$C_{32}H_{38}O_3$. Calculated. C 67.81, H 6.76.
Found, (a). " 67.70, " 6.69.
(b). " 67.70, " 6.83.



STROPHANTHIN.

III. CRYSTALLINE KOMBE STROPHANTHIN—PRELIMINARY NOTE.

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Feist,¹ in 1900, was the first to attempt a careful study of the carbohydrate contained in strophanthin. His material, which was obtained from C. F. Boehringer Sons of Waldhof, was supposedly Kombe strophanthin. The conclusion was reached that the sugar contained in the drug is the methyl ester of a disaccharide of mannose and rhamnose. More recently, in 1913, Brauns and Closson² worked with identified Kombe seeds and were unable to confirm the findings of Feist. On distillation with acid, crystalline Kombe strophanthin as well as several commercial samples failed to give any appreciable amounts of methyl furfural. The conclusion was reached that Kombe strophanthin contains no rhamnose. However, no suggestion was offered as to the real nature of the sugar.

In the year previous to the appearance of the above work, Heffter and Sachs³ published their study of strophanthin prepared both from identified *Strophanthus hispidus* and commercial Kombe seeds. They were unable to prepare an osazone from the strongly reducing sugar fraction obtained from either of these glucosides. For this no explanation was offered.

Following certain preliminary observations we obtained a quantity of seeds from the open market which gave uniformly the green color with sulfuric acid which is regarded as characteristic for the genuine Kombe seed. From this material the crystalline glucoside, which agreed in all properties with those described by the above workers, was readily isolated.

In addition, however, it was found that the substance, when dissolved in acetic acid containing ferrous sulfate and then treated with a few drops of sulfuric acid, quickly yielded a deep purple

¹ Feist, F., *Ber. Chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2069.

² Brauns, D. H., *J. Am. Pharmaceut. Assn.*, 1913, ii, 489, 604. Brauns, D. H., and Closson, O. E., *J. Am. Pharmaceut. Assn.*, 1913, ii, 715.

³ Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83.

solution which, on slight dilution with water, changed to a blue.⁴ The same test was given by a sample of crystalline Kombe strophanthin kindly furnished us by Parke, Davis and Company from the material which had been prepared by Brauns and Closson from identified Kombe seeds. The positive outcome of the Kiliani reaction at once placed the sugar of Kombe strophanthin, in all probability, in the group of the desoxy compounds, digitoxose, and cymarose. This is also supported by the formation of greenish flocks when the sugar solution was boiled with strong hydrochloric acid.

Up to the present, our attempts to prepare the sugar in crystalline form have been unsuccessful. The failure to obtain an osazone is in agreement with the view that the sugar is a desoxy compound. The methyl group in strophanthin is most likely contained in the sugar in the form of methoxyl, as in the case of cymarose, the sugar which Windaus and Hermanns⁵ prepared from cymarín.

Attempts to prepare a crystalline phenylhydrazone or substituted phenylhydrazone have been thus far unsuccessful.

From the formula which we have adopted for strophanthidin, $C_{23}H_{32}O_6$, and on the basis of analytical data obtained with strophanthin itself, it is suggested that crystalline Kombe strophanthin possesses either the formula $C_{32}H_{48}O_{12}$ or $C_{31}H_{46}O_{11}$. In accordance with this the sugar is either $C_9H_{18}O_7$ or $C_8H_{16}O_6$ or the methyl ether of a C_8 or a C_7 desoxy sugar, respectively. An attempt is being made to obtain crystalline derivatives of the sugar.

EXPERIMENTAL.

Commercial strophanthus Kombe seeds which gave uniformly the green color test with sulfuric acid were worked up essentially according to the method used by Brauns and Closson, with the exception that after clearing the solution with basic lead acetate and after subsequent removal of excess lead, the crystalline glucoside was crystallized directly by the cautious addition of ammonium sulfate in an amount just insufficient to salt out the amorphous gummy strophanthin. After several days the crystalline

⁴ Kiliani, H., *Arch. Pharm.*, 1896, cccxxiv, 273; 1913, ccli, 575.

⁵ Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 979.

flocculent glucoside was filtered on a large funnel and washed with small portions of water. It was recrystallized at first by solution in alcohol, addition of an equal volume of water, and then by concentration under diminished pressure. When the alcohol was sufficiently removed, the glucoside crystallized readily as a mass of long, delicate, curved micro needles which were difficult to filter and wash. After several recrystallizations the glucoside was recrystallized directly from water, a process, which, as stated by Brauns and Closson, is accompanied by considerable loss. The glucoside then formed lustrous, long, thin, pointed platelets or when rapidly cooled, long, curved, delicate threads which were often radially grouped.

The substance possessed the recorded properties and melted when rapidly heated at 180–183°, followed by a slow frothing. When a small amount was dissolved in acetic acid and then treated with a few crystals of ferrous sulfate, followed by a few drops of sulfuric acid, the solution gradually developed a deep red-purple color. On addition of a small amount of water, this changed to a blue and finally to green. This color resembles the reaction described by Kiliani for digitoxose and by Windaus and Hermanns for cymarose with the exception that they describe only a blue color. The identical reaction was given by a sample of Kombe strophanthin kindly furnished us by Parke, Davis and Company which had been prepared by Brauns and Closson from identified seed.

$$[\alpha]_{\text{D}}^{25} = 30.5^{\circ} \text{ (} c = 1.02 \text{ in 95 per cent alcohol).}$$

Air-dry substance (dried at 100° in vacuo over H₂SO₄).

C ₃₂ H ₄₈ O ₁₂ ·3H ₂ O.	Calculated.	H ₂ O	7.96.
C ₃₁ H ₄₆ O ₁₁ ·3H ₂ O.	“	“	8.36.
	Found.	“	7.60.

Anhydrous substance.

C ₃₂ H ₄₈ O ₁₂ .	Calculated.	C	61.50,	H	7.75,	OCH ₃	4.97.
C ₃₁ H ₄₆ O ₁₁ .	“	“	62.59,	“	7.80	“	5.22.
	Found, (a).	“	62.02,	“	7.52.		
	(b).	“	62.30,	“	7.57.		
	(c).					OCH ₃	5.26.

0.4051 gm. of anhydrous substance was refluxed for 45 minutes in 25 cc. of 0.1 N NaOH and 25 cc. of water and then titrated against phenolphthalein. 6.7 cc. of alkali were used. Molecular weight is 605. Calculated for C₃₂H₄₈O₁₂ is 624. For C₃₁H₄₆O₁₁ is 594.

5 gm. of crystalline strophanthin were dissolved in a mixture of 40 cc. of 50 per cent alcohol and 10 cc. of concentrated hydrochloric acid and allowed to stand 4 to 6 hours in the cold. The mixture was then diluted and allowed to stand with occasional rubbing. 2.7 gm. of strophanthidin were obtained. The mother liquor was neutralized with pure barium carbonate and the filtrate concentrated under reduced pressure to dryness. The residue was extracted with alcohol and the filtrate from barium chloride was concentrated to dryness. On dissolving the syrup in water an additional 0.3 gm. of strophanthidin was recovered, the total yield amounting to 3 gm. The theory for C_{31} or C_{32} is roughly 3.2 to 3.3 gm.

In the sugar solution, the residual Cl ions were removed with silver sulfate and the filtrate was then treated with hydrogen sulfide. Sulfuric acid, remaining in the filtrate, was removed quantitatively with barium carbonate. The filtrate yielded on concentration a dextro-rotatory syrup which still possessed a somewhat bitter taste. This syrup could not be made to crystallize and attempts to prepare an osazone were fruitless. Likewise, a crystalline phenylhydrazone, bromophenylhydrazone, and benzylphenylhydrazone could not be obtained. The reducing substance was partly soluble in ether and benzene and from its tendency to become resinous on standing, indicated its instability at least in the impure state.

OBSERVATIONS ON DOGS WITH EXPERIMENTAL PYLORIC OBSTRUCTION.

THE ACID-BASE EQUILIBRIUM, CHLORIDES, NON-PROTEIN NITROGEN, AND UREA OF THE BLOOD.

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The present paper reports a short series of experiments on seven dogs with pyloric occlusion and represents a continuation of researches conducted in this laboratory 2 years ago (1). It was of special interest to find (*a*) whether the pH of the blood was increased, (*b*) whether there was any quantitative relationship between the fall in plasma chlorides and the rise in bicarbonate which was known to occur, and (*c*) whether the increased concentration of nitrogen catabolites discovered in human cases of pyloric stenosis (2) occurred in dogs.

With this end in view venous and arterial blood samples were collected before and at periods after operation and the following determinations made: plasma pH, CO₂ and chlorides, and whole blood urea, and non-protein nitrogen.

Procedure.

Large mongrel dogs in apparent good health were selected as subjects. The operation of pyloric occlusion consisted in five cases of a piece of tape tied tightly around the outside and in two cases of complete closure affected by cutting across the pylorus between clamps and inverting each end with a double row of silk ligatures. In three dogs fine rubber tubes with olivary tips of metal (so called duodenal bucket tubes) were sewed into the wall of the stomach and duodenum and then brought out through the flank. By this

device the contents of the stomach and duodenum could be removed and analyzed at will. Water could also be easily introduced into the duodenum. This proved to be a more satisfactory method of supplying fluid than that of hypodermoclysis used in the other dogs. As well as could be judged by hematocrit readings and the absence of thirst, dry tongue, and other objective signs of dehydration, a proper fluid balance was maintained. The dogs refused all food, however, so that the known effects of starvation must be considered in the interpretation of the results. The dogs were observed continuously throughout the day and during part of the night. Arterial blood was collected in some instances from the carotid at the commencement of the operation when the dog was under ether and through the skin without an anesthetic in those cases where a preliminary operation of carotid transplantation had been performed. The venous blood was collected from the jugular with minimum stasis in a Luer syringe lined with pure mineral oil and immediately introduced into small special test-tubes of Pyrex glass under a layer of oil. Potassium oxalate was used as an anticoagulant when such was needed. In all cases the pH and CO_2 determinations were made immediately after centrifugalization of the samples, and the plasma used for chloride estimations was pipetted off at the same time to prevent Cl ion migration from the red cells.

Technique.

The hydron concentration of the plasma was estimated by Cullen's new colorimetric method (3). Glassware and solutions were carefully tested for their possible effects on the pH and all the other details of the method as described were followed. At the time the experiments were performed the method had not been published and the necessary correction constant had not been determined. In calculating our results the following equation was used: $\text{pH}_{35^\circ} = \text{pH}_{t^\circ} + 0.01(t^\circ - 20^\circ) - 18$, where t° = room temperature. The constant (18) has since been found to vary with the species. This number is probably very nearly right if calculating values for rabbit plasma. The correct figure for dog plasma is not yet available and although it seems probable that it is larger than 18 if one can judge from the constant for dog serum, it does not seem advisable to recalculate all the results

using some other tentative value for the constant. This change would not affect the relative value of our analyses which are concerned with a comparison between the pH before and after operation. The method was found eminently satisfactory and the results were even more uniform than we were led to expect from examination of the normal series published by Cullen.

The plasma CO_2 content was measured on the small Van Slyke gas analysis apparatus and all the modifications recently advocated by its designer were followed (4). The BHCO_3 figures were calcu-

lated from the logarithmic formula: $\text{pH} = \text{pK}_1 + \log \frac{[\text{BHC}\text{O}_3]}{[\text{H}_2\text{CO}_3]}$,

where $\text{pK}_1 = 6.10$.

Whole blood chlorides were estimated by the method of Myers and Short (5). The solutions used were of different strengths than those described. The AgNO_3 solution was made up so that 1 cc. was equivalent to 2.5 mg. of NaCl . This solution is much like that of Wetmore (6). The NH_4SCN solution was so prepared that 1 cc. was equivalent to 1 mg. of NaCl ; *i.e.*, 1 cc. of AgNO_3 solution was equivalent to 2.5 cc. of NH_4SCN . This stronger thiocyanate solution gave a much sharper end-point than the solution advocated by Myers and Short. For the plasma chlorides the same standard solutions were used. A saturated picric acid solution was chosen instead of the dry solid picric acid employed in the determination of whole blood chlorides. 2 cc. of oxalated plasma were precipitated by 15 cc. of saturated picric acid and made up to 40 cc. with 23 cc. of water. This was filtered and to a 20 cc. aliquot of the filtrate (equivalent to 1 cc. of plasma) 5 cc. of standard AgNO_3 solution were added, precipitating the chlorides. This was allowed to stand half an hour and then titrated without preliminary removal of AgCl as in the whole blood method. Calculations were made as above.¹

The method of Bell and Doisy (7) with a few modifications was used for tissue chlorides. A simple aspiration apparatus was used in place of the more elaborate one described by the authors. This consisted of the open end of a test-tube drawn out and fused to a glass tube. The test-tube was chosen so that it would fit neatly with a minimum air space into the mouth of a Pyrex combustion

¹ This method is by Dr. Dana Atchley, unpublished.

TABLE I.

Date.	Arterial or venous.	Cells in whole blood.	Plasma Btl.	Total plasma CO ₂ .	Plasma BHC ₂	Plasma BCL	Whole blood urea.	Whole blood non- protein nitrogen.	Remarks.
Experiment 1.									
		per cent		vol. per cent	millimols	gm. per l.	millimols	gm. per 100 cc.	
May 5	V		7.39	52.2	22.2	6.70	111.6		Before operation.
" 8	A		7.40	46.0	19.5	6.81	116.5		At start of operation. Twitching.
" 9	V		7.43	78.3	33.4	5.68	97.2		2 hrs. after mild convulsion. Under anesthesia.
" 10	V		7.47	76.4	32.7	4.97	85.0		
" 11	V		7.41	81.2	34.6	4.18	71.5		
" 11	A		7.49	73.0	31.3	3.66	62.7		
Experiment 2.									
May 6	V		7.35	54.5	23.0	6.65	113.7		Before operation.
" 9	A		7.44	51.9	22.1	6.51	111.5		"
" 12	V		7.42	86.3	36.7				
" 13	A		7.49	77.6	33.3	4.53	77.5		
" 15	V		7.46	91.1	39.0				
" 17	V	52	7.45	87.3	37.3	2.19	37.5	73.5	
" 18	A	50	7.52	76.7	32.7	4.08	69.8	56.6	Preceding mild tetany.
" 24	V		7.44	70.9	30.4	3.92	67.1		
			7.52	90.2	38.5	4.34	74.2		
				88.3	38.0	4.76	81.5		
Experiment 3.									
May 15	V	36	7.42	56.2	24.0	6.58	112.8	42.1	Before operation.
" 17	V	50	7.44	69.6	29.6	6.33	108.2	43.0	
" 18	V		7.41	75.7	32.2	5.82	99.7		

Experiment 4.

May 15	V	38	7.41	56.2	23.9	6.60	113.1	26.6	26.1	Before operation.
Operation, May 15. Complete pyloric occlusion with gastrostomy.										
" 16	V	49	7.40	63.4	20.9	6.32	108.2	32.0	31.0	
	V	53	7.24	64.7	20.9			92.0	90.2	Shortly before death.

Experiment 5.

May 22	V	33	7.38	47.7	20.2	6.66	113.8	32.0	31.0	Before operation.
Operation, May 22. Partial pyloric occlusion. Gastrostomy and duodenostomy.										
" 23	V	36	7.45	65.3	27.9	5.97	102.1	32.0	31.0	
" 24	V	37	7.44	68.4	29.2	5.34	91.4	60.0	52.0	
" 25	V	36	7.52	86.4	37.2	4.64	79.4	88.0	60.0	
" 26	V	37	7.47	93.0	39.8	4.10	70.2			Tetany.
" 28	V		7.56	131.0	56.5	2.5	42.8		135.0	
	A		7.62	122.4	53.0					

Experiment 6.

June 24	V		7.40	51.0	21.7	6.50	111.1	45.6	42.0	Before operation.
Operation, June 24. Partial pyloric occlusion. Gastrostomy and duodenostomy.										
" 26	V	38	7.38	77.8	33.0	4.74	81.1	16.8	38.0	
" 27	V	39	7.39	83.5	35.4	4.23	72.4	29.0	43.0	
" 28	V	38	7.45	91.5	39.1	3.50	59.9	64.4	95.3	
" 29	V							265.6	221.3	At death.

Experiment 7.

June 25	V	41	7.40	64.0	27.2	6.26	107.1	17.0	32.6	
" 26	V	40	7.40	54.8	23.3					
Operation, June 26. Partial pyloric occlusion. Gastrostomy and duodenostomy.										
" 28	V					4.0	68.5	134.2	136.0	At death.

tube (such as is used for doing non-protein nitrogen by Folin's method). The glass tubing was bent so that the test-tube end could be readily introduced into the tilted combustion tube, the other end fitting the absorption tube. This simple apparatus can be easily washed out at the end of the combustion process and meets the requirements of Bell and Doisy that the aspirated fumes shall come into contact with nothing but glass until the HCl is absorbed in the first absorption tube. Comparative blood chloride estimations by this combustion method and by the precipitation method gave accurate checks.

The analyses for urea were made on 3 cc. of whole blood according to Van Slyke and Cullen's modifications (8) of the Marshall method (9), using an alcoholic solution of jack bean and a concentrated buffer solution according to Folin and Wu (10). The method of Folin and Wu (10) was used for the non-protein nitrogen.²

RESULTS.

The figures for plasma pH, CO_2 , chlorides, whole blood urea, and non-protein nitrogen are summarized in Table I.

Of the seven dogs three developed objective signs of hyper-irritability with mild convulsive seizures which we have come to recognize as tetany. In these dogs the pH was definitely increased. In the other dogs (with the exception of Dog 4 who died shortly after operation in acidosis) there was also an increase in the alkalinity of the blood, but to a lesser degree. In every case a rise in CO_2 occurred after operation. The changes in the three dogs which developed tetany may perhaps best be visualized by equating BHCO_3 with pH on coordinate paper (Fig. 1) as done by Van Slyke (11). The parallelogram in the figure includes all normal values found in the seven dogs before operation. Its sides are parallel to a corresponding figure drawn by Van Slyke to represent the range of normality in man, but is made smaller to fit the values found in dogs. It will be seen that the first result of the operation was a shift of the blood into the area designated as compensated alkali excess, but that later the acid-base bal-

² We should like to express our thanks to Mrs. Keeler who made all the urea, non protein nitrogen, and phosphorus analyses in the clinical laboratories of the Presbyterian Hospital.

ance became upset and a condition of uncompensated alkalosis supervened.

Together with the marked rise in CO_2 there was a still greater fall in chlorides. This result after pyloric occlusion has been

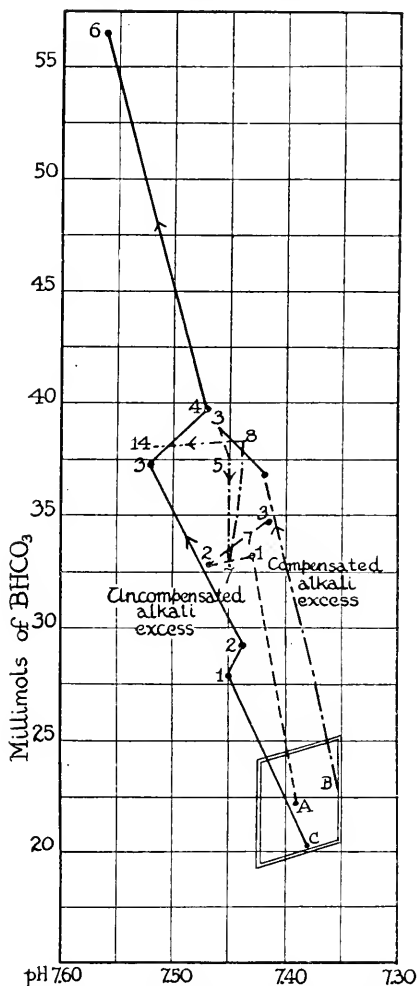


FIG. 1. The abscissæ represent pH of the plasma, the ordinates plasma BHCO_3 in millimols. The parallelogram drawn with a double line marks the limits of normality as determined by preoperative analyses on seven dogs.

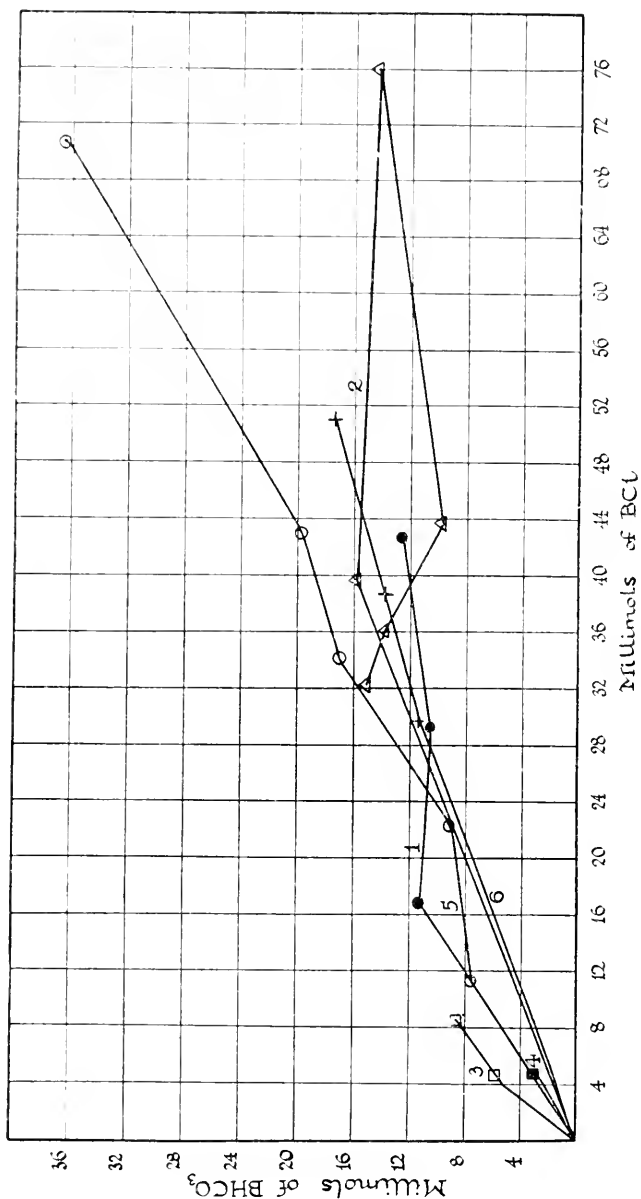


Fig. 2. The abscissa represent the loss of BHC0_3 in millimols and the ordinates the loss of BHC0_3 in millimols after operation. The preoperative value is then 0, and the postoperative changes in each dog are designated by separate lines appropriately numbered.

previously noted both in this and other laboratories. We were interested, however, to see whether there was an inverse quantitative relationship between the two. That there was some relationship, but that the two were not inversely proportional, *i.e.* that each Cl ion lost through the stomach was not replaced by a HCO_3 ion, may be judged from Fig. 2 in which the gain of BHC_3 in millimols is plotted against the loss of BCl in millimols.

That the amount of chloride secreted into the stomach and lost to the body by vomiting or lavage is sufficient to explain the fall in

TABLE II.

Comparative Analyses of Chlorides in Gastric Juice and Blood Plasma.

Dog No.	Time.	Gastric juice.				Blood plasma.	
		0.1 N HCl per 100 cc.		Total H ions.	Total loss of Cl ions through the stomach.	Loss of Cl ions per liter of plasma.	Total loss of Cl ions* in blood plasma.
		Free.	Total.				
				milli-mols	milli-mols	milli-mols	milli-mols
5	First 24 hrs. postoperative.	30.0	35.0	27.1	45.1	11.7	10.0
	Second 24 " "	30.0	42.0	29.4	64.7	10.7	9.3
	Third 24 " "	14.0	24.0	28.8	64.7	12.0	10.3
6	First 54 " "	3.0	5.0	9.5	45.5	38.7	27.6
7	" 19 " "	2.0	3.0	2.6	11.0	4.6	2.7

* Total loss of chloride ions in the blood plasma during the period under consideration was estimated from the preceding column on the basis that the blood plasma constituted 5 per cent of the body weight.

plasma chlorides, was suggested by the gastric analyses of MacCallum and coworkers (12), and is further substantiated by the appended estimations made on the gastric juice removed through the tube which was sewed into the stomach (Table II).

The figures for Dog 5 show consistent results; those for the other two dogs are of little value. Together they indicate that the loss of chlorides through the stomach per day was greater than the drop in plasma chlorides. It must be supposed that the discrepancy represents Cl ions extracted from the tissues. That this is the correct conclusion is suggested by the following chloride

analyses done on pieces of rectus muscle removed at operation and at death, respectively. (Table III.)

Probably the chlorides in the red blood cells are likewise diminished. The figures in Table IV show that in Dogs 6 and 7 the percentage fall in chloride concentration in plasma was approximately the same or only slightly greater than in whole blood.

In the gastric analyses recorded it will be noticed that the mols of Cl ions are far in excess of those of H ions. This we suspect is due to the large admixture of slightly alkaline saliva which contains an appreciable amount of chloride.

TABLE III.
Tissue Chlorides.

Dog No.		At operation.	5 days later.
6	NaCl in mg. per 100 gm. muscle.	319.7	244.3

TABLE IV.
Whole Blood and Plasma Chlorides Compared.

Dog No.	Date.	NaCl per liter of plasma.	Loss.	NaCl per liter of whole blood.	Loss.
		<i>millimols</i>	<i>per cent</i>	<i>millimols</i>	<i>per cent</i>
6	June 24	111.1		91.9	
	" 28	59.9	46		
	" 29			48.8	48
7	" 25	107.1		87.1	
	" 28	68.5	36	51.3	41

In a former paper the increase in sulfate and phosphate ions in the blood plasma was recorded. A few estimations for phosphorus by the method of Doisy and Bell (13) gave the results shown in Table V which substantiate earlier findings.

A study of the recorded non-protein nitrogen and urea values shows that there is a marked rise after pyloric occlusion, the exact cause of which is uncertain. In a number of patients with pyloric stenosis the same finding was repeatedly made. We are inclined to look for a cause to the high bicarbonate values and a possible effect of this change on the kidneys, rather than the possible absorption of toxic nitrogen compounds from the intesti-

nal tract. The urine was not studied chemically, but albumin and casts and an acid reaction were found on several occasions despite the alkaline blood. The contents of the stomach were frequently washed out, and below the obstruction cathartics were several times introduced if the bowels did not move, so that the possibility of absorption from stagnating intestinal contents could be reduced.

Because of the reported electrocardiograph changes in human subjects (14) with alkalosis, tracings of four dogs were taken before and after operation. No consistent changes could be identified in the form or wave intervals to suggest abnormal function. It was noticed, however, that the resistance of the tissues between the electrodes after the salt solution was allowed to soak the skin until no further decrease occurred was over three times as great in the dogs after operation. This is of interest in view of the

TABLE V.
Phosphorus (Mg. per 100 Cc. of Plasma).

Dog No.	Before operation.	After operation.			
		2 days.	3 days.	4 days.	5 days.
5				5.7	
6	3.6	4.9	8.5		16.8
7	3.9	11.0			

marked decrease in the salt content of blood and tissues. In a case of human tetany reported recently (2) the serum conductivity was found to be very low. This aspect of the problem needs further study.

DISCUSSION.

The results outlined give an indication of the disturbance in the acid-base balance which occurs after pyloric obstruction. The slight rise in pH noted in previous experiments, which was believed at the time to be of little consequence, is again reproduced in these experiments and in the three dogs which developed tetany was of sufficient magnitude to make it seem highly probable that there is a causal relationship between diminished acidity and nerve hyperirritability. Greenwald (15) has recently summarized the evidence against the theory that a rise in pH is effective in tetany

production. We believe that the question is still an open one. There is much indirect evidence, for instance, the studies of Grant and Goldman on hyperpnea, that changes in hydron concentration have a definite influence on the irritability of nerve. As regards gastric tetany more facts leading to the same conclusion have been presented in this paper. The essential cause of the disturbance seems to be the excretion of H ions and Cl ions by way of the stomach. Cl ions lost from the blood seem to be replaced in a measure by other anions; bicarbonate, phosphate, and sulfate ions etc. As there are more Cl ions than H ions secreted in the salivary and gastric juices together, the loss of Cl ions and gain in BHCO_3 ions in the blood are not strictly proportional inversely.

Further studies on nitrogen metabolism and the effect of alkalies on renal function will have to be made before it becomes clear why there is an increase of the nitrogen waste products in the blood.³

SUMMARY AND CONCLUSIONS.

In seven dogs in whom complete or partial pyloric stenosis was effected the following changes occurred after operation:

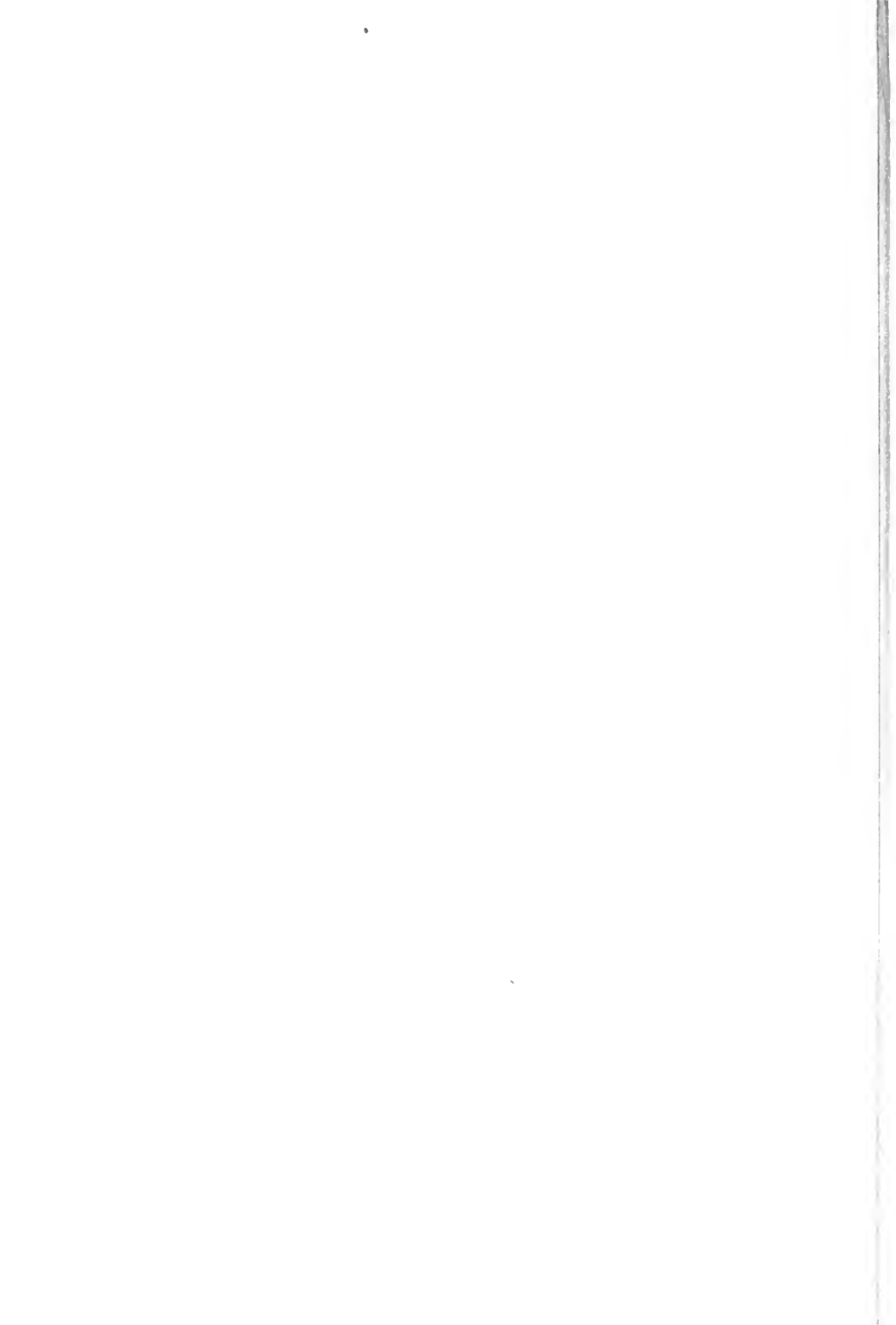
1. A rise in pH, which, however, was only marked in the three dogs that developed tetany.
2. A marked increase in bicarbonate concentration.
3. A very rapid diminution of chlorides in the plasma, whole blood, and tissues which could be accounted for by the amount of chloride secretion into the gastrointestinal tract above the point of obstruction.
4. An inconsistent but in some cases a marked rise in urea and non-protein nitrogen of whole blood.

Mention was also made of an increased concentration of phosphorus in two dogs and an augmented electrical resistance noted while taking electrocardiograms in four dogs. (The electrocardiogram was in each case negative.)

³ Since the completion of this writing two articles have appeared in the literature dealing with this question (16, 17). The results are in accord with our findings, but in some instances the suggested interpretations were dissimilar.

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CLINICAL CALORIMETRY.

XXXIII. THE EFFECT OF FASTING IN DIABETES AS COMPARED WITH A DIET DESIGNED TO REPLACE THE FOODSTUFFS OXIDIZED DURING A FAST.

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INTRODUCTION.

The outstanding achievement in the treatment of diabetes, the development of a non-toxic extract of the internal secretion of the pancreas by Banting and others (1, 2) in the laboratory of MacLeod (3), was published during the progress of the work described in this paper. The success of this extract appears to be conditioned mainly on the possibility of preparing it on the huge scale that will be required as soon as diabetics are kept alive solely by its use. The great prevalence of diabetes makes it unlikely that

dietetic measures will be abandoned. Further study of the diet leads, moreover, to conclusions of physiological and practical interest.

During a fast a diabetic patient continues to oxidize foodstuffs derived from his own tissues. Why not substitute for this food material an identical quantity given in the diet? There is no *a priori* reason why this change from endogenous to exogenous foodstuffs should have any effect on the metabolism, either in the total calories or in the proportions of protein, fat, and carbohydrate oxidized. If the effect is nil, reduction of the intake of food below the level of the fasting metabolism merely wastes the tissues without benefit to the patient. This statement needs qualification only if the waste of tissue is beneficial in itself and not in its effect on the metabolism. The method used in this investigation was first to determine by means of the respiration calorimeter the quantity of protein, fat, and carbohydrate oxidized while fasting, and to give the same quantities in the diet. This diet we have referred to as the *replacement diet*. The effect of fasting as opposed to this diet was observed, using as a basis for comparison the total metabolism and the quantities of protein, fat, and carbohydrate oxidized. The data were also used to make a comparison between the food ingested and the food oxidized, in order to strike a balance after the manner of the nitrogen balance, not only for protein, but for carbohydrate and fat as well.

Literature.

The total metabolism in diabetes was extensively studied in 1910 by Benedict and Joslin (4). The earlier literature was fully reviewed by them. They found an increase in the heat production, whereas Allen and Du Bois (5) found no increase, the disagreement being caused by the difference in the standards used to determine the normal metabolism. Wilder, Boothby, and Beeler (6) recently investigated the total metabolism in thirty-one cases of uncomplicated diabetes, and found a depression roughly proportional to the depression of the weight below the medico-actuarial standards.

The increase in the total metabolism following the ingestion of food is known as the specific dynamic action of foodstuffs, and varies with the kind of food ingested. Protein causes the greatest rise in metabolism, fat and carbohydrate much less (7). Experiments on the specific dynamic action have been limited as a rule to observing the effect of large quantities of food. The effect of quantities not more than sufficient to replace those

oxidized during a fast has been very little investigated. To one dog, which while starving oxidized 96 grams of fat Voit gave 100 grams, with the result that it oxidized 97 grams (8). In the twenty-sixth paper of this series, Soderstrom, Barr, and Du Bois (9) observed the effect of a small breakfast on the heat production of normal individuals. "The standard breakfast contained approximately 222 calories, enough to supply the basal energy requirement of the subjects three to four hours." It contained 4.7 gm. of protein, 9.0 gm. of fat, and 28.9 gm. of carbohydrate. "In the first hour following the ingestion of this the heat production increased on an average 7 per cent.; in the second hour, 2 per cent.; in the third hour 2 per cent." It was therefore possible to give food somewhat above the basal requirements (for 2 hours) without a sustained rise in the metabolism.

Benedict and Carpenter (10) working with normal individuals, have computed the "cost of digestion," which is related to the specific dynamic action, but is expressed differently. It is the absolute, instead of the percentage increase in the heat production following a meal. With mixed meals Benedict and Carpenter find that the cost of digestion averages 6 per cent of the fuel value of the intake. The meals which they used were heavy. For a replacement diet the increase in metabolism of a normal individual would not therefore be expected to exceed 6 per cent of the fuel value of the intake, which in this case is the same as the basal metabolism.

Wilder, Boothby, and Beeler (6) in the course of a recent study of a severe diabetic, observed the effect, on the metabolism, of a mixed meal containing little or no carbohydrate, a variable amount of protein, and a large proportion of fat. The least rise in the metabolism was observed with a diet which contained 1,007 calories per 24 hours, as compared to a basal metabolism of 910 calories, and was therefore 11 per cent above the basal requirements. One-third of this was given just before the observation, and consisted of 6.5 gm. of protein, 31.4 gm. of fat, and 4.1 gm. of carbohydrate. The metabolism increased from -16 per cent of the average normal just before the meal to +1 per cent afterwards, and remained well above basal at the end of 3 hours. Food thus given in the customary three meals, though only 11 per cent above the basal requirements sufficed to cause a sharp and sustained rise in metabolism.

Method.

Seven patients, all with mild or moderately severe diabetes, were observed in the respiration calorimeter of the Russell Sage Institute of Pathology. The technique was the same as previously described (11, 12). The patients were kept under close observation in a small metabolism ward, as described in Paper III of this series (13). Observations were controlled by means of alcohol checks.

In Table I the theoretical quantities, as calculated from the quantity of alcohol burned, are compared with the quantities

Feb. 16	79.66	1	7.82	55.38	54.13	-2.3	16.30	15.83	-2.9	14.94	14.60	-2.3	11.17	11.45	+2.5	0.671
		2	7.69	54.42	53.84	-1.1	16.02	15.93	-0.6	14.69	14.57	-0.8	10.98	11.39	+3.7	0.665
		3	7.62	53.95	55.70	+3.2	15.88	16.00	+0.8	14.56	14.53	-0.2	10.89	11.48	+5.4	0.661
		Average.	7.71	54.58	54.56	± 0.0	16.07	15.92	-0.9	14.73	14.57	-1.1	11.01	11.44	+3.9	0.666
Apr. 8	79.66	1	7.49	53.00	51.98	-1.9	15.61	15.70	+0.6	14.31	13.91	-2.8	10.70	11.83	+10.6	0.644
		2	7.42	52.53	52.11	-0.8	15.47	15.10	-2.4	14.18	13.79	-2.7	10.60	11.33	+6.9	0.664
		3	7.42	52.53	52.08	-0.9	15.47	15.45	-0.1	14.18	13.90	-2.0	10.60	11.48	+8.3	0.654
		Average.	7.44	52.69	52.06	-1.2	15.52	15.42	-0.6	14.22	13.87	-2.5	10.63	11.55	+8.7	0.654
Apr. 13	79.66	1	8.15	57.74	55.09	-4.6	17.00	16.40	-3.5	15.58	15.28	-1.9	11.65	12.13	+4.1	0.678
		2	7.89	55.81	55.10	-1.3	16.44	16.51	+0.6	15.07	15.21	+0.9	11.27	11.87	+5.3	0.669
		3	7.95	56.32	54.90	-2.5	16.58	15.89	-4.2	15.20	14.86	-2.2	11.37	11.82	+4.0	0.680
		Average.	8.00	56.63	55.03	-2.8	16.67	16.28	-2.3	15.28	15.12	-1.0	11.43	11.94	+4.5	0.676
Total average.....			7.70	54.26	53.98	-0.5	15.98	15.73	-1.6	14.65	14.48	-1.1	10.95	11.42	+4.3	0.6696

Jan. 10, 1922. 47.9 kg. 1.59 sq.m.	11.43 a.m. to 1.43 p.m.	16.1	15.2	0.77	0.581	49.6	52	6	Food at 2 hr. intervals; last meal at 10 a.m. Moved twice, asleep 40 min., quiet.
Jan. 13, 1922. 46.5 kg.	12.35 p.m. to 2.35 p.m.	15.1	13.8	0.79	0.365	45.7	54	9	Fasting. Quiet, asleep 20 min. Turned over.
Jan. 14, 1922. 47.6 kg. 1.58 sq.m.	11.24 a.m. to 1.24 p.m.	15.8	14.3	0.80	0.603	47.0	55	8	Food at 2 hr. intervals; last meal at 10 a.m. Quiet.
Chas. C. Jan. 20, 1922. 49.2 kg. 1.52 sq.m.	11.44 a.m. to 1.41 p.m.	14.7	14.4	0.75	0.382	46.9	64	9	Fasting. Quiet.
Jan. 21, 1922. 49.4 kg. 1.52 sq.m.	11.28 a.m. to 1.28 p.m.	15.6	15.5	0.73	0.470	50.3	62	5	Food at 2 hr. intervals; last meal at 10 a.m. Quiet, slept 20 min.
Jan. 23, 1922. 49.7 kg. 1.53 sq.m.	11.35 a.m. to 1.35 p.m.	15.6	15.4	0.73	0.640	49.8	64	11	Food at 2 hr. intervals; last meal at 10 a.m. Quiet, asleep 50 min.
Jan. 24, 1922. 49.0 kg. 1.52 sq.m.	11.18 a.m. to 1.18 p.m.	14.0	13.3	0.77	0.765	42.9	72	4	Fasting. Very quiet. Asleep most of 1 hr.
Jan. 25, 1922. 49.7 kg. 1.53 sq.m.	11.37 a.m. to 1.37 p.m.	14.6	13.8	0.77	0.373	45.3	72	6	Food at 2 hr. intervals; last meal at 10 a.m. Quiet, asleep 20 min.

* The work adder is an instrument by which the activity of the patient is measured. A very quiet patient raises the work adder less than 5 cm., and a very restless patient more than 25 cm.

TABLE II—*Continued.*

Subject. Date. Weight. Surface area.	Time of observation.	CO ₂ <i>ccm.</i>	O ₂ <i>ccm.</i>	R.Q.	N ₂ in urine.	Indirect calories.	Average pulse.	Work, adder ² , (average) <i>cm.</i>	Remarks.
Ben. J. Feb. 6, 1922. 39.2 kg. 1.43 sq.m. Feb. 7, 1922. 39.9 kg.	12.31 p.m. to 2.34 p.m.	13.2	11.2	0.86	0.291	37.6	54	4	Fasting. Quiet, voided once.
	11.42 a.m. to 1.12 p.m.	13.5	10.8	0.91	0.538	36.2	53	5	Food at 2 hr. intervals; last meal at 10 a.m.
	12.17 p.m. to 2.17 p.m.	12.0	10.5	0.83	0.545	34.6	62	5	Quiet, asleep 25 min. Fasting. Very quiet.
	11.30 a.m. to 2.30 p.m.	12.3	10.5	0.86	0.672	34.4	59	4	Food at 2 hr. intervals; last meal at 10 a.m.
	12.00 m. to 3.00 p.m.	12.3	10.9	0.79	0.610	35.5	59	5	Very quiet. Food at 2 hr. intervals; last meal at 10 a.m.
Frank C. Feb. 21, 1922. 51.8 kg. 1.59 sq.m. Feb. 23, 1922. 52.2 kg. 1.59 sq.m.	11.20 a.m. to 1.20 p.m.	15.9	14.7	0.79	0.486	48.3	59	5	Fasting. Quiet.
	11.49 a.m. to 2.49 p.m.	17.3	15.4	0.82	0.530	50.8	54	8	Food at 2 hr. intervals; last meal at 10 a.m. Quiet.

Feb. 28, 1922. 51.9 kg. 1.59 sq.m.	11.46 a.m. to 2.46 p.m.	17.1	15.0	0.83	0.451	49.9	56	7	Food at 2 hr. intervals; last meal at 10 a.m. Quiet, though somewhat rest- less during last hr.
Patrick M. Apr. 14, 1922. 60.4 kg. 1.74 sq.m.	12.22 p.m. to 2.22 p.m.	18.2	15.7	0.85	0.321	52.7	60	6	Fasting. Quiet.
Apr. 15, 1922. 60.8 kg. 1.74 sq.m.	11.13 a.m. to 1.13 p.m.	19.3	16.5	0.85	0.349	55.1	61		Food at 2 hr. intervals; last meal at 10 a.m. Quiet.
Frank B. Apr. 19, 1922. 41.9 kg. 1.43 sq.m.	11.47 a.m. to 1.47 p.m.	20.5	19.9	0.75	1.045	64.3	68	18	Breakfast 10 a.m. Restless.
Apr. 20, 1922. 43.2 kg. 1.44 sq.m.	11.43 a.m. to 1.43 p.m.	16.2	15.7	0.75	0.498	51.0	62	20	Fasting. Restless.
Apr. 21, 1922. 43.7 kg. 1.45 sq.m.	11.47 a.m. to 1.47 p.m.	15.4	14.6	0.76	0.632	47.7	61	11	Continued fast. Quiet.
Apr. 25, 1922. 45.4 kg. 1.48 sq.m.	12.07 p.m. to 2.07 p.m.	16.9	14.8	0.83	0.578	49.0	64	5	Food at 2 hr. intervals; last meal at 10 a.m. Quiet.
Apr. 26, 1922. 42.5 kg. 1.41 sq.m.	11.58 a.m. to 1.58 p.m.	14.8	13.4	0.80	0.345	44.3	58	10	Fasting. Fairly quiet.

TABLE II. *Concluded.*

Subject. Date. Weight. Surface area.	Time of observation.	CO ₂ .	O.	R.Q.	N. in urine.	Indirect calories.	Average pulse.	Work added* (average).	Remarks.
Apr. 28, 1922. 42.9 kg. 1.45 sq.m.	11.58 a.m. to 1.58 p.m.	16.1 gms.	14.0 "	0.84	0.712	46.1	60	5 cm.	Breakfast at 6 a.m. Quiet.
Chas. S. Apr. 5, 1922. 47.4 kg. 1.53 sq.m.	12.09 p.m. to 1.34 p.m.	22.9	22.8	0.73		74.8†	92	23	No food. Semicomatose, but very restless at times. Voided.
Apr. 6, 1922. 46.4 kg. 1.52 sq.m.	11.47 a.m. to 2.47 p.m.	20.2	20.4	0.72		66.9†	99	12	50 gm. glucose at 10.50 a.m. Quiet.

† Approximate indirect.

observed, and the percentage error of the observation is indicated. For heat the error averaged -0.5 per cent, for oxygen -1.6 per cent, for carbon dioxide -1.1 per cent, and for water $+4.3$ per cent. The conclusions of this paper are based on the oxygen consumption and the respiratory quotient. The maximum range of error in a 3 hour period was, in the case of oxygen, from $+0.3$ to -2.4 per cent. The respiratory quotient ranged from a minimum of 0.654 to a maximum of 0.680 and averaged 0.670 as against the theoretical quotient 0.667 .

For our purpose the ideal technique would have been to keep the patient under observation in a chamber calorimeter for the entire 24 hours, giving the diet in the usual three or four meals. This method was not available. The period during which an observation can be continued with profit is often limited even in the respiration calorimeter, and for this reason it was necessary to find some means by which a period of 2 or 3 hours could be made a fair sample of the 24 hours. This was done simply by giving the diet in small portions every 2 hours, and making the calorimeter observation as soon as possible after the last meal. It was thought that by this means fluctuations both in the absorption and metabolism of food would be minimized. Although the results were calculated as for 24 hours, the period both of the diet and of the observation was only 2 or 3 hours. The meal which had an immediate influence on the metabolism was presumably the last one before the observation, that is, the 10 a.m. meal, the previous meals serving merely to bring the patient into equilibrium. There was, therefore, no need of allowing for the movement of the patient about the ward, as he was kept in bed for at least 12 hours before the observation. As the ward is small the amount of energy which the patients are able to expend is in any case slight. The diet was given every 2 hours, except for two periods of 4 hours each beginning at 10 p.m. and 2 a.m., when a double portion was given. It was begun at the latest 4, 6, or 8 p.m. of the day before, and sometimes two or more days previous. In the fasting observations food was taken not later than noon of the preceding day, sometimes of the day before that.

The determination of the urinary nitrogen was done by the Kjeldahl method; and glucose by the Benedict (14) method. Total acetone bodies of the urine were estimated by the method

of Van Slyke (15) and expressed as acetone. The blood sugar of the whole blood was estimated by the method of Benedict (16), and the carbon dioxide-combining power of the blood by the method of Van Slyke and Cullen (17) and Van Slyke (18).

Seven patients were studied, besides the one in coma. An observation on each of two additional patients was discarded because of flaws in the technique.

Case Histories.

All the patients had mild or moderately severe diabetes, with the exception of one patient who was in diabetic coma. Complications were absent.

Case 1.—James F., age 52 years, porter, born in the United States, was admitted to the metabolism ward on Dec. 11, 1921. He had no symptoms of diabetes, and glycosuria was detected only on routine examination. His blood sugar decreased from 472 mg. per 100 cc. to 200 mg. His maximum excretion of acetone bodies, on a protein-fat diet was 3.31 gm. His tolerance for carbohydrate as measured clinically reached 150 gm.

Case 2.—Moderate diabetes. Francis M., age 23 years, chauffeur, born in the United States, was admitted to the metabolism ward on Jan. 3, 1922. The onset of diabetic symptoms was in 1919. His blood sugar on Jan. 3, 1922, was 214 mg. per 100 cc. His maximum excretion of acetone bodies was 1.92 gm. on a protein-fat diet. He developed a tolerance for 80 gm. of carbohydrate.

Case 3.—Moderately severe diabetes. Chas. C., age 53 years, laborer, born in Bohemia, was admitted to the metabolism ward on Jan. 18, 1922. He began to have symptoms of diabetes in Jan., 1920. Apart from loss of flesh and arteriosclerosis his physical examination was negative. His blood sugar ranged from 291 to 236 mg. per 100 cc. His maximum excretion of the acetone bodies was 4.19 gm. on a protein-fat diet. He developed a tolerance for 60 gm. of protein and 10 gm. of carbohydrate.

Case 4.—Moderately severe diabetes. Ben. J., age 16 years, schoolboy, born in the United States, was admitted to the metabolism ward on Feb. 2, 1922. His symptoms of diabetes dated from May, 1920. Examination revealed extreme emaciation, a flushed face, rapid respiration, and a red tongue. His blood sugar ranged from 210 to 362 mg. per 100 cc. He excreted a maximum of 14.3 gm. of acetone bodies on a high fat diet. He died of erysipelas on May 4, 1922.

Case 5.—Mild diabetes. Frank C., age 27 years, accountant, born in the United States, was admitted to the metabolism ward on Feb. 6, 1922. His symptoms began in Jan., 1921. His blood sugar was 210 mg. per 100 cc. on Feb. 7. His excretion of acetone bodies never exceeded 1 gm. He developed a tolerance for 83 gm. of carbohydrate.

Case 6.—Mild diabetes. Patriek M., age 46 years, policeman, born in Ireland, was admitted to the metabolism ward on Apr. 10, 1922. His diabetes was detected in Oct., 1921. The physical examination was negative except for signs of disease of the posterior roots and peripheral nerves due to diabetes. His blood sugar was 85 mg. per 100 cc. on Apr. 3, 1922. He developed a tolerance for 125 gm. of carbohydrate.

Case 7.—Moderately severe diabetes. Frank B., age 24 years, condiment maker, born in Austria, was admitted to the metabolism ward on Apr. 17, 1922. His symptoms began 6 or 8 months before admission. Physical examination showed him to be undernourished, flushed, with acetone odor of the breath. His blood sugar ranged from 422 to 130 mg. per 100 cc. The maximum excretion of the acetone bodies was 2.27 gm. On Apr. 21 a boil about 4 cm. in diameter was incised and 4 cc. of pus evacuated. He developed a tolerance for 100 gm. of carbohydrate.

The following patient is included as an example of the metabolism in diabetic coma.

Case 8.—Chas. S., age 39 years, carpenter, born in Finland, was admitted to the metabolism ward on Apr. 5, 1922. His symptoms of diabetes began 10 weeks previously. Physical examination showed him to be undernourished, stuporous, flushed, with dry tongue, Kussmaul breathing, and acetone odor of breath. Recalculated for a 24 hour period the urine from 9.30 p.m., Apr. 5 to 3.40 a.m., Apr. 6 contained 9.07 gm. of acetone and diacetic acid, 55.8 gm. of β -hydroxybutyric acid, 24.25 gm. of nitrogen, and 102 gm. of glucose. The blood sugar expressed in mg. per 100 cc. was 400 on Apr. 5, the date of the first calorimeter observation; 476 on Apr. 6, before the second observation; and 716 on the afternoon of the same day, shortly before death. The carbon dioxide-combining power of the blood was 18 volumes per cent before the observation and 17 after it. He received 15 to 20 gm. of sodium bicarbonate and 3 to 5 liters of fluid daily. He received 25 gm. of glucose daily for 2 days, and on the 3rd, 50 gm. before entering the calorimeter. The calorimeter data are shown in Table II. During the first observation the respiratory quotient was 0.73. On the 2nd day, after receiving the glucose the quotients for the first 3 hours were 0.74, 0.72, and 0.70, respectively, and averaged 0.72, the same as on the previous day. The carbon dioxide-combining power of the blood remained unchanged, and the blood sugar increased. There was no evidence that any of the glucose was oxidized. The only effect on the respiratory quotient was a definite depression.

Results of Experiments.

I. Basal Metabolism in Diabetes.

In Table III the third column indicates the weight of the patient, and the fifth column the average normal weight for persons of the same age and height, derived from the medico-actuarial tables as published by Joslin (19). The tables were

TABLE III.
Body Weight and Basal Metabolism.

Name.	Date.	Age.	Height.	Weight.	Surface.	Average normal weight.	Per-centage change in weight.	Calories per square meter.	Normal metabolism per square meter.	Per-centage change in metabolism.	Total acetone bodies.	Urine glucose.	Urine nitrogen.
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
			<i>cm.</i>	<i>kg.</i>	<i>sq. m.</i>	<i>kg.</i>					<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
James F.	Dec. 16	52	167	83.3	1.92	65	+28	40.7	37.5	+9	3.31	0	13.1
Francis M.	Jan. 5	23	177	47.7	1.59	66	-28	31.7	39.5	-20	0.93	0	11.9
Charles C.	Jan. 20	53	166	49.2	1.52	65	-24	30.8	37.5	-18	1.14	16.6	11.3
Ben. J.	Feb. 6	16	173	39.2	1.43	59	-34	26.3	43.0	-39	0.003	0	8.9
Frank C.	Mar. 4	27	171	51.8	1.59	64	-19	30.4	39.5	-23	0.403	0	11.0
Patrick M.	Apr. 14	46	174	60.4	1.74	71	-15	30.5	38.5	-21	0.064	0	8.07
Frank B.	Apr. 26	34	166	42.5	1.44	62	-31	30.8	39.5	-22	0.200	Trace.	9.15
Charles S.	Apr. 6	39	170	46.1	1.52	66	-30	44.0	39.5	+11	33.01	102	24.3

corrected by allowing 1 inch for heels of shoes, and 10 pounds for clothes. Results are expressed in kilos, net. The sixth column represents the deviation from the average, expressed as a percentage. The ninth column indicates the deviation of the metabolism from the normal standards of Aub and Du Bois (20), also expressed as a percentage. This is also known as the basal metabolic rate. Change in weight closely paralleled change in metabolism. In Fig. 1 the same data are plotted, the abscissæ repre-

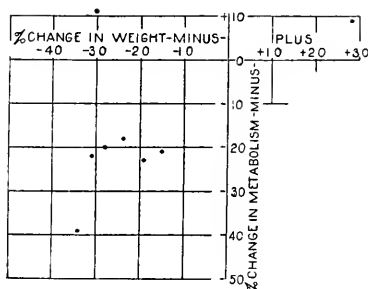


FIG. 1. Total metabolism and weight. The abscissæ represent increase or decrease in weight expressed as a percentage of normal; the ordinates, increase or decrease in metabolism expressed as a percentage of normal. Each dot represents one observation. Eight patients were observed.

senting the percentage loss in weight, and the ordinates the percentage loss in metabolism. All but one of the observations are placed in such a way as to indicate a rough proportion between loss of weight and depression of the metabolism. The exception, shown in the figure with a metabolism of +11 and a weight of -30 per cent, occurred with the patient who was in diabetic coma. From the distribution of the other dots, a metabolism of -25 per cent would have been expected. The cause of this high level will be discussed later, but it may be mentioned that this patient was oxidizing protein at a very rapid rate, as shown by the excretion of 24.3 gm. of nitrogen per 24 hours. In the other cases the relation of metabolism to weight is sufficiently close to a proportion to suggest that the depression of the metabolism is due to undernutrition and not to any inherent peculiarity of the disease.

TABLE IV.
Food Eaten Compared with Food Oxidized per 24 Hrs.

Date	Diet calories.				Metabolized calories.			
	Protein.	Fat.	Carbo- hydrate.	Total.	Protein.	Fat.	Carbo- hydrate.	Total.
James F.								
Dec. 16	0	0	0	0	294	1,399	184	1,877
" 17	246	1,406	0	1,652	459	1,320	118	1,897
Francis M.								
Jan. 5	0	0	0	0	288	613	312	1,213
" 6	295	621	0	916	466	601	112	1,209
" 7	296	619	0	915	452	731	91	1,279
" 10	305	1,058	0	1,363	372	683	137	1,191
" 13	0	0	0	0	232	611	253	1,097
" 14	305	591	0	896	384	499	246	1,128
Charles C.								
Jan. 20	0	0	0	0	243	824	57	1,124
" 21	301	826	0	1,126	299	901	6	1,206
" 23	300	1,237	0	1,537	407	789	0	1,196
" 24	0	0	0	0	487	500	43	1,029
" 25	300	502	0	802	237	691	159	1,088
Ben. J.								
Feb. 6	0	0	0	0	185	305	413	904
" 7	183	304	414	901	342	11	516	869
" 13	0	0	0	0	347	253	230	830
" 17	346	428	60	834	428	114	284	825
" 20	346	848	60	1,254	388	348	115	851
Frank C.								
Mar. 21	0	0	0	0	309	631	220	1,160
" 23	310	628	221	1,160	337	538	345	1,220
" 28	308	1,209	221	1,738	287	495	415	1,196
Patrick M.								
Apr. 14	0	0	0	0	204	523	538	1,265
" 15	206	525	541	1,272	221	520	588	1,330
Frank B.								
Apr. 21	0	0	0	0	402	651	88	1,141
" 25	491	660	89	1,150	368	422	383	1,172
" 26	0	0	0	0	220	565	278	1,063

II. Effect of Replacement Diet Compared to Fasting.

a. On Total Metabolism.—The results are shown in Table IV. On the left is indicated in calories the diet per 24 hours divided

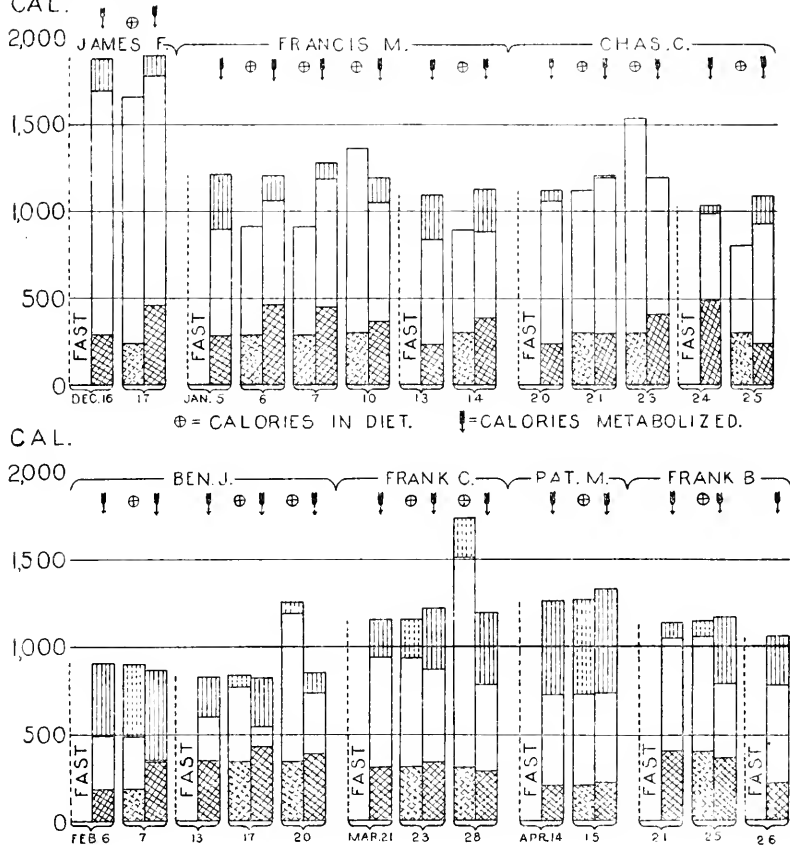


FIG. 2. Comparison of food eaten and oxidized, in terms of calories per 24 hours. The total height of the columns represents the total calories of diet or metabolism. The cross-hatched portion at the bottom represents protein, the middle portion, which is blank, represents fat, and the upper portion, vertically shaded, represents carbohydrate. The diet is marked by a circle, and further, by broken shading. When no food was given the fact is indicated by the word "fast." The calories metabolized are designated by arrows.

into protein, fat, and carbohydrate. The first three patients received 1.5 gm. of protein per kilo of body weight, fat equal to the

quantity oxidized while fasting, and no carbohydrate. The others received a diet designed to replace all food elements oxidized while fasting. Both groups received on occasion an excess of fat above the basal requirements. On the right of the table is indicated the calories metabolized, also divided into protein, fat, and carbohydrate. Fig. 2 presents the same data in graphic form. The results are summarized in Table V, in which the figures denote the increase or decrease in the calories metabolized per 24 hours with the diet, as compared to those metabolized while fasting. In the fifth column the increase or decrease is expressed as a percentage. The greatest increase in metabolism as a result of a protein-fat diet was observed in the case of Chas. C., Table IV, January 20 and 21. The metabolism increased from 1,124 calories per 24 hours during the fast to 1,206 calories with the diet, an increase of 7.3 per cent. The least change was in the case of Francis M. on January 6. The metabolism decreased from 1,213 to 1,209 calories per 24 hours, a decrease of 0.3 per cent. The average increase for the three cases was 3.9 per cent. In the remaining four patients the diet consisted of the amounts of protein, fat, and carbohydrate which were oxidized during the fast. The greatest rise in metabolism was in the case of Patrick M., April 14 and 15. The metabolism increased from 1,265 to 1,330 calories per 24 hours, or 5.1 per cent. The least change was in the case of Ben. J., February 6 and 7. The metabolism decreased from 904 to 869 calories per 24 hours. The average increase for this group was 1.7 per cent. The average increase in total metabolism over fasting for the series was 2.6 per cent. Thus it was found possible to give to a patient as many calories as he would have utilized while fasting with no significant rise in metabolism. That the small extent of the rise was not due to lack of calories in the diet was proved by giving extra fat, in the sense of fat in excess of the quantity which had been oxidized by the patient while fasting. This excess amounted to from 30 to 230 per cent. Francis M. on January 10 received over 50 per cent excess and his metabolism remained even below the fasting level. Frank C. on March 28 received 100 per cent in excess and his metabolism increased only 3.1 per cent. The average increase for the patients who received the excess of fat was only 2.5 per cent above the level observed during the previous

fast. The increase was thus essentially the same as it had been with the replacement diet alone. These results hold for a diet continued for 4 days.

The extension of the fast to 2 days and of the diet to between 2 and 7 days caused no greater increase in metabolism than did the shorter periods. Thus the increase in metabolism, as compared to that of the previous fast, averaged 2.6 per cent for 1 day of fast and 1 day of diet, and only 1.7 per cent for 2 days of fast and 2 or more days of diet.

The effect of a second fast is shown in Table IV and Fig. 2, in the case of Francis M. on January 13, Chas. C. on January 24, Ben. J. on February 13, and Frank B. on April 26. The decrease

TABLE V.

Increase or Decrease in the Oxidation of Foodstuffs When the Replacement Diet is Substituted for Fasting.

Diet.	Calories oxidized per 24 hours.				
	Absolute increase or decrease.				Percentage increase.
	Protein.	Fat.	Carbo- hy- drate.	Total.	Total.
Protein-fat diet.....	+74	+2	-43	+33	+3.9
“ “ + additional fat.....	+124	+17	-116	+25	+2.3
Replacement diet.....	+50	-152	+125	+23	+1.7
“ “ + additional fat....	+10	-20	+40	+29	+2.8
Averages.....	+66			+30	+2.6

in the metabolism as compared to that of the previous diet was 7.9, 14.0, 4.5, and 9.3 per cent, respectively; an average decrease of 9 per cent. The decrease in metabolism as compared to that of the previous fast was 9.5, 8.5, 8.2, and 6.8 per cent, respectively; an average decrease of 8.3 per cent. It may be said then, that the replacement diet held the metabolism at about the level of the previous fast, but did not cause any decrease, such as resulted from a second fast.

b. Effect of Replacement Diet on the Metabolism of Protein, Fat, and Carbohydrate.—Results are shown in Tables IV and V, and in Fig. 2.

The protein metabolism increased somewhat with the replacement diet and also in three out of the four patients to whom excess

of fat was given. The increase for the entire series averaged 16 gm. a day (66 calories). The metabolism of carbohydrate and fat depended on whether or not the carbohydrate of the fast was replaced. When the carbohydrate of the fast was replaced, as was done with the last four patients, they oxidized more carbohydrate than after 24 hours of fast. In such cases the metabolism of fat decreased. When protein and fat alone were replaced, as was done with the first three patients, they oxidized less carbohydrate than during a fast. The metabolism of fat then increased, but only to an insignificant extent, averaging 2 gm. a day.

When carbohydrate was replaced glycosuria appeared or increased. Thus Ben. J. had no glycosuria after a fast on February 13, but excreted 5 gm. on February 17 after 3 days of replacement diet. Frank B. had only a trace of glucose in the urine on the 2nd day of fasting, but excreted nearly 20 gm. a day for 3 days of replacement diet. This increase in glycosuria associated with an increase in the metabolism both of protein and carbohydrate with the replacement diet suggests an increased mobilization of glucose.

III. Comparison of Food Ingested with Food Oxidized.

This paper has dealt so far with the behavior of the metabolism under different conditions. The data have been utilized in another way, to compare the quantity of food eaten with the quantity oxidized. This is an extension of the idea of balance as conveyed by the term nitrogen balance. The latter is the relation between the quantity of protein ingested and the quantity oxidized. The same conception may be applied to carbohydrate and fat, provided that allowance be made for any portion which escapes oxidation and passes into the urine. These balances were determined in observations which lasted 2 or 3 hours.

In Table IV, comparison of Columns 1 and 5 gives the protein balance. The results are shown graphically in Fig. 2. The protein ingested is seen in nearly every case to be less than the protein oxidized.

The carbohydrate balance is similar to the protein balance. The first three patients of Table IV received no carbohydrate, yet they all oxidized carbohydrate. For at least 6 consecutive days Francis M. continued to oxidize carbohydrate: 76 gm. on the 1st day, and 33 gm. on the 6th. Chas. C. oxidized 11 gm. on the 5th

day and 39 gm. on the 6th. Frank C. at the end of 2 days of complete fasting oxidized 54 gm. Frank B. at the end of 2 days oxidized 21 gm. It is evident that whether fasting completely, or merely foregoing carbohydrate, these patients continued to draw on their own tissues for considerable quantities.

With respect to the fat balance, there is a relation diametrically different from that which obtains with the other two foodstuffs. There is evidence of a very marked storage of fat. For example, Francis M., on January 10, oxidized 73 out of 114 gm.; Chas. C., on January 23, oxidized 85 out of 133 gm.; Ben. J., on February 20, oxidized 37 out of 91 gm.; Frank C., on March 28, oxidized 53 out of 130 gm. The tissues of these undernourished diabetics seem to soak up fat like a sponge. Ben. J., who was emaciated to the last degree, on February 7, stored the entire amount given, and oxidized protein and carbohydrate instead.

DISCUSSION.

I. Technique.

It is to be noted that the results obtained in this paper were based upon observations after 12 hours of complete rest. The conclusions might be modified if there were an increase in the activity of the patient combined with an alteration in the proportions of foodstuffs oxidized. For the present we are unable to apply them strictly except to patients at rest.

An average of $1\frac{1}{2}$ hours elapsed between the last meal and the beginning of the calorimeter observations. The results were, however, the same in later observations in which this period was included.

II. Results.

Basal Metabolism.—Seven of the patients were amenable to treatment by means of the diet and may be said to have been under control, in the sense that they had neither marked ketosis nor severe glycosuria. Another patient was in diabetic coma. The basal metabolism of the seven patients whose diabetes was under control was found to be depressed in rough proportion to the percentage diminution of the weight below the average of the medico-actuarial standards. The lower the weight the lower the

metabolism. The results tend to confirm those previously obtained in this laboratory, and those of Wilder, Boothby, and Beeler (6). On the other hand, the metabolism of a patient in coma was conspicuously in excess of that to be expected of a man of his age and weight. This excess tends to bear out the contention of Benedict and Joslin (4) that there is an increase in the metabolism in cases of acidosis. There are several factors which might be responsible for the increase, but most of it could be accounted for on the basis of the increase in the metabolism of protein. Using the table of Aub and Du Bois (21) we calculated that 7.6 calories per hour were due to his metabolism of protein over and above the average of the other patients. His total metabolism of 44 calories per hour -7.6 leaves 36.5, which divided by an estimated normal of 39.5 gives 92 per cent, or -8 instead of $+11$ per cent. Another factor which may have increased the metabolism of this patient was his extremely labored respiration.

Metabolism with Replacement Diet.—The metabolism rose with the replacement diet only slightly above the basal level, on the average 2.6 per cent. This diet seemed to hold the metabolism at the level previously reached by fasting, although considerably above the level which resulted from a subsequent fast. Had the same amount of food been given in larger meals at less frequent intervals a larger rise might have been expected. On the one occasion when we tried this the metabolism rose only 5 per cent above the fasting level.

Effect of the Oxidation of Protein, Fat, and Carbohydrate.—Though the metabolism as a whole changed little with the replacement diet the oxidation of the individual food elements was not the same as it had been during the fast. The oxidation of protein increased. When the diet contained no carbohydrate the oxidation of carbohydrate fell off and the glucose in the urine remained stationary. When, on the other hand, the diet did contain carbohydrate the oxidation of this substance increased, and with it the glycosuria, showing that the patient's ability to oxidize glucose had been taxed. To remedy this defect the allowance of protein and carbohydrate should be curtailed, and the fat correspondingly increased. The result would resemble the diets advocated by Newburgh and Marsh (22). Experiments with this type of diet are to be reported from this laboratory later.

Comparison of Food Ingested with Food Oxidized.—A quantitative comparison between the food ingested and oxidized revealed a negative protein balance, a negative carbohydrate balance, and a markedly positive fat balance. The negative protein balance is similar to the negative nitrogen balance frequently observed in diabetes, and requires no comment. The negative carbohydrate balance is of interest. It means that to the diet as given the tissues may contribute a quantity which is, in the absence of respiratory data, unknown. Even if this added carbohydrate has a beneficial effect it contains an element of danger in that it might suddenly fail. Another interesting question arises as to its source. Certainly it cannot come from the original store of glycogen present in health, which is estimated by Joslin (23) to average 400 gm., and is hardly sufficient to last the month out at the rate at which we have observed it to be withdrawn. Hence there must be at times storage of carbohydrate in order that at other times there may be withdrawal. Where this storage can occur is a puzzle, because the diabetic has been considered very poor in glycogen. Perhaps this depletion may be exaggerated, as indicated in an interesting discussion by Joslin (23).

In computing the fat balance no deduction was made for fat in the feces. A liberal allowance for this factor would be 10 per cent of the intake (24, 25). As none of our patients had diarrhea, and most were constipated, no reason is apparent for their exceeding this quantity. Even with this allowance there was still a very markedly positive fat balance.

We found no limit in the ability of our patients to store fat. The largest quantity of fat given was 120 gm. and even with this amount the metabolism of fat was no greater than it had been during the fast.

SUMMARY AND CONCLUSIONS.

1. By means of the respiration calorimeter the quantities of protein, fat, and carbohydrate oxidized by diabetic patients at rest were determined. The same amounts were then given in the diet, which we have called a replacement diet. With this the metabolism was held at the level previously reached by fasting.

2. According to evidence based on the respiratory quotient, the replacement diet was attended with an increase in the quantity

of protein and carbohydrate oxidized, and of glucose excreted. To remedy this defect it should be modified by decreasing the protein and carbohydrate, and increasing the fat, thus approaching the Newburgh type of diet.

3. A method is described for the quantitative comparison of the food ingested with the food oxidized. Diabetic patients, receiving their basal caloric requirements plus in several instances considerable quantities of fat, oxidized more protein, more carbohydrate, and less fat than they received. In other words, to their allowance of food they added protein and carbohydrate derived from their own tissues, and subtracted a considerable portion of the fat for storage.

4. It is inferred that the quantities of food eaten by diabetic patients may be widely different from the quantities oxidized, and that calculations based on the diet are subject to error.

5. The above results apply strictly only to diabetic patients at rest.

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THE APPLICATION OF THE QUINHYDRONE ELECTRODE TO ELECTROMETRIC ACID-BASE TITRATIONS IN THE PRESENCE OF AIR, AND THE FACTORS LIMITING ITS USE IN ALKALINE SOLUTION.*

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As the title indicates, this paper deals with the limits of applicability of a simplified method for the determination of hydrogen ion concentration, suggested by Biilmann, in which the hydrogen electrode is replaced by an electrode, using a solution of benzoquinhydrone in place of gaseous hydrogen, and more particularly with the causes underlying the limitations of this method in alkaline solution.

It is well known that the hydrogen electrode does not give satisfactory results in the presence of atmospheric oxygen, unsaturated substances, and traces of other active oxidizing agents such as nitrate ions. It is possible, of course, to use the hydrogen electrode when such interfering substances are present by permitting the solution to remain in contact with the platinum black hydrogen combination long enough so that the system is reduced to such a point that it will support the oxidation-reduction potential set up by one atmosphere of hydrogen. This procedure, however, is not always feasible, and when it is the true hydrogen ion concentration of the original solution may

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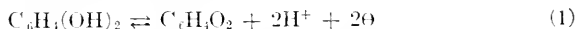
be altered on account of the removal of hydrogen ions in the reduction process or simply as a result of the change in the acidic or basic dissociation constants that is created by the reduction of one part of the molecule of the acids or bases that are present.

The change in the hydrogen ion concentration of blood owing to the removal of oxygen is an important instance of the latter case and it was partly with the idea of later investigating the possibility of devising a method for determining the hydrogen ion concentration directly in oxygenated blood that these experiments were undertaken.

In addition to possessing the advantage of enabling one to determine pH very accurately in the presence of certain of the oxidizing agents mentioned above, the benzoquinhydrone method possesses the further advantage of simplicity in that it eliminates the need for a supply of hydrogen and its attendant purification.

The technique consists simply in dissolving a few crystals of this quinhydrone in the medium under examination and measuring the resulting oxidation-reduction potential, which is promptly established with an inert electrode such as plain, or better, gold plated platinum in the usual manner by opposing it to a calomel half cell.

The theory of the method is based upon the studies of Haber and Russ (2) and of Granger and Nelson (3) who have shown that benzoquinhydrone in aqueous solution is highly dissociated into its equimolecular components quinone and hydroquinone which are further in equilibrium with one another according to the following electrochemical equation:



Since the reaction is strictly reversible Peters' form of the van't Hoff isotherm is applicable and we have for 25°C. the relation

$$\pi = \pi_0 + \frac{0.059}{2} \log \frac{[\text{C}_6\text{H}_4\text{O}_2]}{[\text{C}_6\text{H}_4(\text{OH})_2]} + 0.059 \log \text{H}^+ \quad (2)$$

Here π is the electrode potential observed and π_0 is the normal potential of the system; namely, +0.6990 volts on the hydrogen electrode scale. From this it is seen that the electrode potential (which is determined by the electron pressure, or better, the

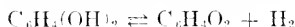
electron activity of the solution) is a linear function of the logarithm of the hydrogen ion concentration as long as reaction (1) remains uncomplicated by side reactions.

Bühlmann (4), working independently of Granger and Nelson, was the first to advocate the method as a means of determining pH. He pointed out that since benzoquinhydrone yielded equimolecular quantities of quinone and hydroquinone the term involving the concentration ratio under such conditions vanishes and we have

$$-\log H^+ = \text{pH} = \frac{\pi_0 - \pi_{\text{observed}}}{0.059} \quad (3)$$

an equation exactly analogous to the usual hydrogen electrode equation except for the value of π_0 .

Bühlmann derives this equation from a somewhat different view-point. He considers the following equilibrium to be set up:



so that

$$[\text{H}_2] = K \frac{[\text{C}_6\text{H}_4(\text{OH})_2]}{[\text{C}_6\text{H}_4\text{O}_2]}$$

Here $[\text{H}_2]$ equals $10^{-23.65}$ atmospheres when the ratio hydroquinone to quinone is unity as is the case with quinhydrone, and the quinhydrone electrode possesses a partial (thermodynamic) pressure of $10^{-23.65}$ atmospheres of hydrogen which he calls P_q .

From the equation connecting two hydrogen electrodes at pressures of one atmosphere and P_q atmospheres we get:

$$\text{Pressure correction} = \frac{0.059}{2} \log \frac{1}{P_q} = + 0.6993 \text{ volts}$$

as the correction to be applied to a hydrogen electrode under the reduced pressure of P_q atmospheres.

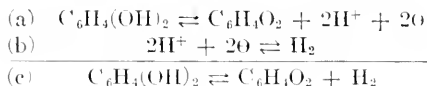
Substituting this correction in the equation

$$\text{pH} = \frac{\pi_0 \text{ hydrogen} - \pi_{\text{observed}} + \text{pressure correction}}{0.059}$$

we obtain equation (3) since the normal potential of the hydrogen electrode (π_0 hydrogen) is by definition considered as zero.

That is, we may say that we are supplying hydrogen in solid form at a constant infinitesimal value from the dissociation pressure of the hydroquinone of the quinhydrone.

This is equivalent to assuming that the two following electrochemical reactions (a) and (b) are in mobile equilibrium so that we have their sum (c) as an active reaction:



The two view-points, of course, are of equal value thermodynamically as neither the time factor nor the path by which equilibrium is reached is concerned in a thermodynamic derivation.

The present writers believe, however, that the electrochemical view-point which seeks to correlate all oxidation-reduction potentials as being due to an effective electron pressure¹ is more in accord with the kinetics of the reaction than the one which considers the electrode to function as a hydrogen electrode at low partial pressure. The reasons for our preference are as follows:

1. A calculated partial pressure of $10^{-23.65}$ atmospheres of hydrogen is so vanishingly small that it cannot represent an actual concentration, but is simply a measure of the probability that electrons and hydrogen ions are combined at any given moment as hydrogen molecules. This probability would correspond to a concentration represented by 1.4 molecules of hydrogen gas in 22.4 liters of gas surrounding the electrode, a quantity insufficient to be of any statistical importance.

2. Experiments have shown that gaseous hydrogen will not reduce quinone in the absence of platinum black, and a plain gold electrode immersed in a quinhydrone solution saturated with hydrogen will give the pH corresponding to the quinone-hydroquinone equilibrium. The fact that the gold electrode reacts promptly to changes in pH, but not to changes in hydrogen pressure, indicates very strongly, in our opinion that reaction (b) and therefore reaction (c) are not in mobile equilibrium so that it is only reaction (a) which should be considered.

¹ See particularly Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922, where this view-point has been developed in more detail.

Some recent work of Conant and Cutter² may be interpreted as indicating that reversible reductions like quinone to hydroquinone consist in the simultaneous addition of hydrogen ions and electrons while irreversible reductions such as the saturation of a double bond require the electron and hydrogen ion to be combined first as a hydrogen atom and then added to the double bond.

We may consider then that it is both this inertness of reaction (b) in the absence of platinum black as well as the extremely low partial pressure of hydrogen which may be considered to be the reason why Biilmann had success with the electrode in the presence of unsaturated organic substances. It will be highly important to investigate the rate at which such oxidizing and reducing substances affect hydroquinone and quinone for the rational extension of the electrode to other systems.

Some preliminary experiments on the use of the quinhydrone electrode as an alternative to the hydrogen electrode, carried out by one of us in the summer of 1922 yielded results similar to those given in Fig. 1 where the pH values obtained in the titration of acids by bases are plotted against the quantity of alkali added, using both the quinhydrone and hydrogen electrodes.

These experiments showed at once that the quinhydrone electrode was admirably suited for the determination of the titration values of strong acids since it gave breaks at the end-point comparable to the hydrogen electrode, came to equilibrium very promptly, and above all was more simple to operate with limited equipment.

It was accordingly considered worth while to investigate the electrode more carefully in neutral and alkaline solution than has been previously done, since this is the most important range for biological purposes, as well as of being important for estimating the minimum strength of acids which can be titrated in its presence.

Before proceeding to the experimental data we shall present the various circumstances which may cause the quinhydrone electrode to give values different from the hydrogen electrode.

Equation (2) shows that the validity of the method depends

² Conant, J. B., and Cutter, H. B., *J. Am. Chem. Soc.*, 1922, xlv, 2651.

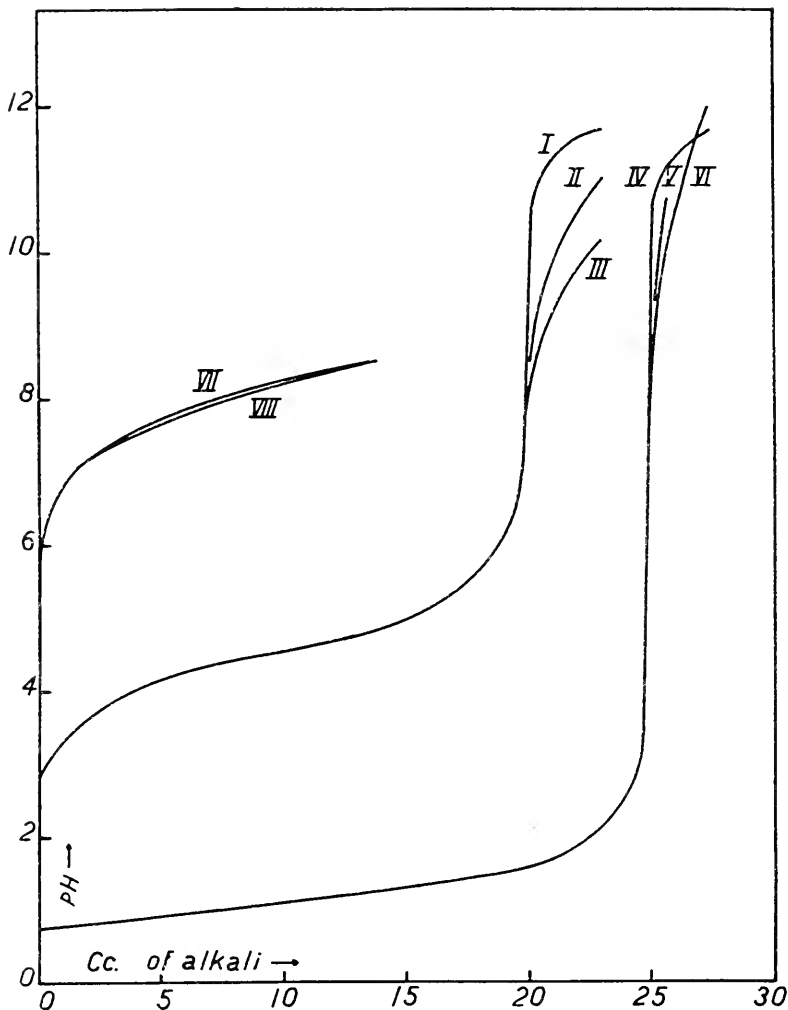


FIG. 1. Curve I represents 0.2 M acetic acid—hydrogen electrode; Curve II, 0.2 M acetic acid—quinhydrone electrode corrected for factor (Ia), see page 623; Curve III, 0.2 M acetic acid—quinhydrone electrode uncorrected; Curve IV, 0.2 M hydrochloric acid—hydrogen electrode; Curve V, 0.2 M hydrochloric acid—quinhydrone electrode corrected for factor (Ia); Curve VI, 0.2 M hydrochloric acid—quinhydrone electrode uncorrected; Curve VII, 0.2 M boric acid titrated with 0.05 M borax—hydrogen electrode; and Curve VIII, 0.2 M boric acid titrated with 0.05 M borax—quinhydrone electrode.

primarily upon maintaining the ratio of quinone to hydroquinone strictly equal to unity or to some other constant and known value.

There are at least three factors which may cause this ratio to be disturbed; namely,

I. The ionization of hydroquinone as a weak acid. This, (Ia), may affect the observed potential by changing the nature of the electrode reaction so that it takes the course indicated by



the reduced form existing as an ion, an equation which yields a potential independent of the hydrogen ion concentration; or (Ib) it may affect the actual pH of the solution by participating in the acid-base equilibrium through removal of hydroxyl ions through neutralization of the hydroquinone in alkaline solution.

II. Through hydroquinone or quinone being converted into the other by the action of some other active oxidizing or reducing component present in the system, or by their unequal conversion into other bodies. The presence of such rapid oxidizing agents as ferric, permanganate, and dichromate ions or the rapid reducing agents as iodide, titanous, and chromous ions, to mention only a few, will obviously interfere with the use of the quinhydrone electrode, as all of these substances react so rapidly with hydroquinone or quinone that they may be used to determine them in volumetric analysis. The autoxidation of hydroquinone by the oxygen of the air is a particularly important practical complication falling under this heading and will be the only one considered in this paper.

III. Through changes in the activities of hydroquinone and of quinone due to the presence of other electrolytes. This is due to the fact that the position of an equilibrium is determined not by the stoichiometrical concentrations of the participating substances but by their thermodynamic concentrations (*i.e.*, their activities or their active mass in the older terminology). The activity of the hydrogen ion does not enter in this case as it is the logarithm of the activity rather than of the stoichiometrical concentration of hydrogen ion which is measured and designated as pH. The activity question has been carefully studied by Sørensen and his collaborators (5) who have shown that the

electrode exhibits a marked salt effect in concentrated solution. Fortunately, the error is not important for the salt concentrations usually met with under physiological conditions, and may be dismissed with the statement that it may be considered to cause an error of less than 0.5 millivolt (pH values 0.01 too large) provided the total salt concentrations involved are less than 0.2 molar. Until further work has been done on other salt systems than hydrochloric acid and sodium chloride, this statement should be considered general only if the ions involved are univalent and do not show a very marked tendency to hydrate in solution.

In contrast to factor (III), the data will show that factors (I) and (II) may become of considerable importance in an alkaline solution.

EXPERIMENTAL.

Careful electrometric titrations furnish the simplest way of obtaining data over a wide range of pH to test the relative importance of the factors enumerated. Parallel titrations were accordingly made, using the quinhydrone electrode in one case and the hydrogen electrode in another. The experiments recorded here were performed in a water bath at 25 and 37.5°C.

The titrating apparatus was practically identical with that employed by La Mer and Baker (6) in their work on the oxidation-reduction potentials of the substituted quinone-hydroquinone systems, except that the inlet and outlet tubes for nitrogen were dispensed with when the quinhydrone electrode was used, free access of air being permitted since it was particularly desired to test what effect air had upon the results with quinhydrone.

A small glass cylinder of approximately 100 cc. capacity served as the titrating vessel and it was supported in the bath so that shaking could be readily obtained after each addition of alkali. Both the hydrogen and quinhydrone electrodes were measured against the saturated calomel half cell, connection with which was made by a salt bridge consisting of a glass tube 4 mm. in diameter and filled with saturated potassium chloride.

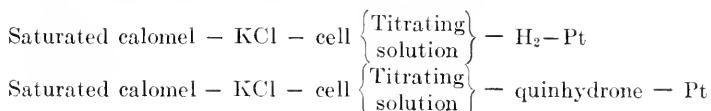
It is highly important for precise electrometric titration work to prevent the interdiffusion of KCl from the salt bridge and the substances which are being titrated in the electrode vessel. This

is necessary not only to prevent escape of the material being titrated but since a contact potential exists, whose magnitude varies with the condition of mixing of the two fluids at the interface, it must be maintained at a constant value throughout the titration.

We have found these requirements to be admirably fulfilled by inserting a small glass stopper into the salt bridge, the stopper being ground sufficiently tight to prevent diffusion and yet afford good conductivity. After a period of 10 to 20 minutes a constant and reproducible contact potential is established showing that a mixing equilibrium exists in the capillaries between the stopper and wall of the glass tube. This contact potential is not disturbed by the small fluctuations in pressure caused by the bubbling of the hydrogen through the solution in the electrode vessel. However, complete interference with the escape of hydrogen so that some fluid is forced through the capillaries owing to the pressure developed, causes a disturbance in the mixing equilibrium with the result that the contact potential may change as much as several millivolts when hydrochloric acid is used.

When this occurs it usually requires another 10 to 20 minutes to reestablish the original contact potential, so that it is necessary to guard against excess pressure in the electrode vessel, else one may be deceived into thinking that electrode equilibrium has not been reached.

The cells measured may be represented as follows:



The observed E.M.F. is recorded as positive when the pole on the right hand side as written above is positive.

A detailed summary of some of the typical results is given in Tables I to IV. The pH values recorded in Columns 4 and 5 of these tables are calculated from equation (3) using the value 0.4520 volt for the quinhydrone electrode when opposed to the saturated calomel cell at 25°C. This follows if we take the saturated calomel cell including its contact potential to be 0.2470 volt positive to the normal hydrogen electrode. This value was

that obtained by La Mer and Baker (6) assuming $0.1 \times \text{HCl}$ to be 92 per cent ionized. Their work is in perfect agreement with that of Bühlmann in that the quinhydrone electrode is 0.6990 ± 0.0002 volt positive to the hydrogen electrode at 25°C . It is immaterial for a comparison of the quinhydrone and hydrogen

TABLE 4.
Electrometric Titration of 20 Cc. of 0.2 M Acetic Acid with Approximately 0.2 M Sodium Hydroxide.

Temperature 25°C . End-point electrometrically 19.60 cc. Phenolphthalein 19.60 cc.

NaOH added. (1)	H ₂ electro- metric calomel. (2)	Quinhydrone calomel. (3)	H ₂ electro- metric. (4)	Quin- hydrone. (5)	Column 3 - Column 2. (6)	Column 5 - Column 4. (7)
cc.			pH	pH		
0.00	-0.4061	+0.2936	2.86	2.68	0.6997	-0.18
2.00	-0.4957	+0.2333	3.71	3.71	0.6990	± 0.00
4.00	-0.4858	+0.2137	4.05	4.04	0.6995	-0.01
6.00	-0.4992	+0.2005	4.27	4.26	0.6997	-0.01
8.00	-0.5102	+0.1891	4.45	4.45	0.6993	± 0.00
10.00	-0.5203	+0.1789	4.63	4.62	0.6992	± 0.00
12.00	-0.5306	+0.1684	4.80	4.80	0.6990	± 0.00
14.00	-0.5435	+0.1556	5.02	5.02	0.6991	± 0.00
16.00	-0.5584	+0.1403	5.26	5.26	0.6987	± 0.00
17.00	-0.5695	+0.1297	5.45	5.45	0.6992	± 0.00
18.00	-0.5824	+0.1140	5.70	5.72	0.6964	+0.02
18.50	-0.5948	+0.1033	5.89	5.90	0.6981	+0.01
19.00	-0.6164	+0.0844	6.25	6.22	0.7008	-0.03
19.40	-0.6703	+0.0449	7.16	6.91	0.7143	-0.25
19.50	-0.7370	+0.0292	8.30	7.16	0.7662	-1.24
19.84	-0.8917	-0.0482	10.90	8.47	0.8435	-2.43
20.00	-0.9693	-0.0715	11.20	8.85	0.8378	-2.35
20.50	-0.9249	-0.1021	11.50	9.36	0.8728	-2.14
21.00	-0.9340	-0.1187	11.63	9.66	0.8153	-1.97
21.50	-0.9404	-0.1320	11.71	9.86	0.8084	-1.85
22.00	-0.9459	-0.1451	11.85	10.10	0.7999	-1.75
22.50	-0.9485	-0.1565	11.88	10.29	0.7920	-1.59

electrodes if the value 0.2470 for the calomel cell is not absolutely correct as such error affects both electrodes equally.

The difference in the observed potentials of the hydrogen and quinhydrone electrodes is given in Column 6 of the tables. Deviations from the theoretical value 0.6990 indicate how much the observed quinhydrone electrode potentials differ from those

of the hydrogen electrode owing to the operation of factors (I) to (III). In Column 7 we have given the discrepancy between the two electrodes in terms of pH units. Since no correction has been made for the salt error in 0.2 molar buffers, a discrepancy of 0.01 pH unit may be expected between the two electrodes. It will be seen from all the tables that the differences in the pH

TABLE II.

Electrometric Titration at 25°C. of 25 Cc. of 0.2 M KH_2PO_4 with 0.2 M NaOH Showing Results Using the Hydrogen Electrode and the Quinhydrone Electrode in the Presence of Air.

NaOH added. (1)	H ₂ electro- metric calomel. (2)	Quinhydrone calomel. (3)	H ₂ electro- metric. (4)	Quin- hydrone. (5)	Column 3— Column 2 (6)	Column 5— Column 4. (7)
cc.			pH	pH		
0.00	-0.5038(?)	+0.1989(?)	4.35(?)	4.28(?)	0.7027(?)	-0.07(?)
2.00	-0.5784(?)	+0.1230	5.61	5.57	0.7014	-0.04
3.00	-0.5899(?)	+0.1111	5.80	5.78	0.7010	-0.02
5.00	-0.6056(?)	+0.0944	6.07	6.05	0.7009	-0.02
7.00	-0.6172	+0.0832	6.27	6.25	0.7004	-0.02
9.00	-0.6271	+0.0725	6.44	6.43	0.6996	-0.01
11.00	-0.6358	+0.0637	6.58	6.58	0.6995	±0.00
13.00	-0.6443	+0.0553	6.72	6.72	0.6996	±0.00
15.00	-0.6530	+0.0473	6.87	6.85	0.7006	-0.02
17.00	-0.6622	+0.0386	7.02	7.00	0.7008	-0.02
19.00	-0.6731	+0.0265	7.20	7.20	0.6996	±0.00
21.00	-0.6871	+0.0127	7.45	7.44	0.6998	-0.01
23.00	-0.7093	-0.0079	7.83	7.79	0.7014	-0.04
24.00	-0.7270	-0.0264	8.13	8.10	0.7006	-0.03
25.00	-0.8179(?)	-0.0604(?)	9.66(?)	8.67(?)	0.7575(?)	-0.99(?)

Potential of the quinhydrone electrode at pH 8.13 stable to 0.2 millivolt for 8 minutes but drifted 2 millivolts in 27 minutes on the last addition of alkali (25.00 cc.). The hydrogen electrode also drifted slightly due no doubt to lack of adequate buffering. The slight discrepancies noted in acid solution were due more to sluggishness on the part of the hydrogen electrodes, than to the quinhydrone electrodes, as the latter checked perfectly among themselves, while the former required a very much longer time for equilibrium.

values do not usually exceed this amount except in alkaline solution where the difference is sometimes considerable, or in those portions of the curve where the pH is not well buffered and consequently easily affected by minute errors in the addition of the alkali. This will be noted only in pure acid solution or when passing through a neutralization end-point. This concordance

TABLE III.

Electrometric Titration of 0.2 M Boric Acid and 0.2 M KCl by 0.2 M NaOH Using Quinhydrone Electrode in the Presence of Air and Hydrogen Electrode at 25°C.

NaOH added. (1)	H ₂ electro- metric calomel. (2)	Quinhydrone calomel. (3)	H ₂ electro- metric. (4)	Quin- hydrone. (5)	Column 3— Column 2. (6)	Column 5— Column 4. (7)
cc.			pH	pH		
0.00	-0.5696	+0.1303	5.46	5.44	0.6999	-0.02
1.00	-0.6496	+0.0500	6.81	6.80	0.6996	-0.01
2.00	-0.6700	+0.0283	7.16	7.17	0.6983	+0.01
4.00	-0.6942	+0.0047	7.56	7.56	0.6989	±0.00
6.00	-0.7105	-0.0117	7.85	7.85	0.6988	±0.00
8.00	-0.7228	-0.0237	8.05	8.05	0.6991	±0.00
9.00	-0.7285	-0.0299	8.14	8.15	0.6986	+0.01
10.00	-0.7338	-0.0353	8.23	8.25	0.6985	+0.01
11.00	-0.7386	-0.0404	8.32	8.34	0.6983	+0.02
13.00	-0.7468	-0.0494	8.45	8.48	0.6974	+0.03
15.00	-0.7542	-0.0579	8.58	8.63	0.6967	+0.05

Quinhydrone electrode exhibited a slow drift at pH 8.14, but was apparently stable at pH 8.05. The time consumed in taking readings from pH 8.14 on, totaled 10 minutes, or an average of 2 minutes per addition.

TABLE IV.

Electrometric Titration at 37.5°C. of 29 Cc. of 0.2 M KH₂PO₄ with 0.2 M NaOH with the Hydrogen and with Quinhydrone Electrode.

Saturated calomel at 37.5°C. (provisionally taken as 0.2445); i.e., (0.2470 - 12.5 × 0.0002 volt). Barometer correction of 0.9 millivolt applied to hydrogen electrode.

NaOH added. (1)	H ₂ electro- metric calomel. (2)	Quinhydrone calomel. (3)	H ₂ electro- metric. (4)	Quin- hydrone. (5)	Column 3— Column 2. (6)	Column 5— Column 4. (7)
cc.			pH	pH		
0.00	-0.5064	+0.1835	4.24	4.26	0.6899	+0.02
1.00	-0.5696	+0.1215	5.26	5.27	0.6911	+0.01
2.00	-0.5896	+0.1009	5.60	5.60	0.6905	±0.00
3.00	-0.6022	+0.0883	5.80	5.80	0.6905	±0.00
4.00	-0.6120	+0.0786	5.96	5.96	0.6906	±0.00
6.00	-0.6270	+0.0637	6.21	6.21	0.6907	±0.00
8.00	-0.6394	+0.0511	6.41	6.41	0.6905	±0.00
12.00	-0.6621	+0.0286	6.77	6.77	0.6907	±0.00
14.00	-0.6744	+0.0165	6.97	6.97	0.6909	±0.00
16.00	-0.6897	+0.0016	7.23	7.21	0.6913	-0.02
18.00	-0.7141	-0.0195	7.63	7.56	0.6946	-0.07
19.00	-0.7398	-0.0378	8.05	7.85	0.7020	-0.20
20.00	-0.8279	-0.0625(?)	9.46	8.25	0.7654	-1.21
21.00	-0.8682	-0.0890(?)	10.10	8.69	0.7792	-1.41

At pH 7.61 quinhydrone potentials commenced to rise on standing. At pH 10.10 the potentials drifted rapidly. The potential of the quinhydrone electrode against the calomel at 37.5°C. is taken provisionally as 0.4460 volt.

in buffered acid solution may be taken as evidence of the correctness of our setting the effect of the salt error at 0.01 pH unit for the buffers investigated.

The magnitude of factor (Ia) can be evaluated from a knowledge of the acidic dissociation constants of hydroquinone by using the more general form of Peters' equation recently derived and tested on some anthraquinone salts by Conant, Kahn, Fieser, and Kurtz (7). These authors have shown that as the alkalinity is increased the electrode reaction gradually changes from the type given in equation (1) over to that given by equation (4) depending entirely upon the extent to which hydrogen ions combine with the hydroquinone ions. Their more general equation is identical with the usual equation (2) except for the addition of a correction factor,

$$\frac{0.059}{2} \log \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2} \right\}$$

K_1 and K_2 are the primary and secondary ionization constants of the hydroquinone. Euler and Bolin (8) found K_1 to be 1.1×10^{-10} from conductivity experiments, while Sheppard (9) from a hydrogen electrode titration gives 1.75×10^{-10} and 4×10^{-12} for K_1 and K_2 , respectively.

It can readily be seen that the entire correction factor vanishes in acid solution where $[H^+]$ is large compared to K_1 and K_2 . In Table V we have given the value of the correction term for alkaline solution, using the larger values of the dissociation constants. The correction is entirely negligible for pH values less than 8.50. In Fig. 1 we have applied the correction to the titration curves of hydrochloric and acetic acids, and while it makes them approximate more closely to the hydrogen electrode curves in alkaline solution, this correction alone is inadequate to account for the discrepancies observed in alkaline solution.

That it is factor (Ib) which is largely responsible for the discrepancy between the curves may be ascertained by a simple calculation. When a weak acid is half neutralized by a strong base the pH of the solution equals $-\log K_1$ or 9.76 for hydroquinone since $-\log 1.75 \times 10^{-10}$ is 9.76. The solubility of quinhydrone is 0.013 molar of which 95 per cent or 0.012 molar is dissociated as hydroquinone. The pH of a pure aqueous

unbuffered solution containing sufficient NaOH to half neutralize this quantity of hydroquinone is 0.003 molar which would correspond to a pH of 11.73 were no hydroquinone present.

The difference (11.73 - 9.76) 1.97 represents the change in pH owing to the participation of hydroquinone in the acid-base equilibrium at this pH.

TABLE V.

Table Showing the Value of the Correction Factor $\frac{0.059}{2} \log \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2} \right\}$ To Be Applied to the Quinhydrone Electrode to Take Account of the Ionization of Hydroquinone with Consequent Change in the Nature of the Electrode Reaction.

pH	$\frac{0.059}{2} \log \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2} \right\}$	Apparent pH given by quinhydrone electrode.	Correction in pH units.
7.60	0.0001	7.60	+0.002
8.00	0.0002	8.00	+0.003
8.50	0.0007	8.49	+0.01
8.80	0.0012	8.78	+0.02
9.00	0.0023	8.96	+0.04
9.30	0.0038	9.23	+0.07
9.50	0.0056	9.40	+0.10
9.80	0.0090	9.65	+0.15
10.00	0.0133	9.77	+0.23
10.30	0.0200	9.96	+0.34
10.50	0.0254	10.07	+0.43
11.00	0.0413	10.30	+0.70
11.50	0.0620	10.45	+1.05

The correction factor $\frac{0.059}{2} \left\{ \log 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2} \right\}$ amounts to but 15 millivolts at this pH or a total correction of 0.131 volts for factors (Ia) and (Ib) which is more than sufficient to account for the discrepancy in the two curves. Further evidence that factor (Ib) is the one largely responsible in the case of the hydrochloric and acetic acid curves in the unbuffered alkaline range may be seen from Table IV where the two electrodes check very well up to pH 8.58 in borate mixture which is heavily buffered in this range and consequently prevents any marked change in actual pH due to factor (Ib). Another experiment using 0.05 M borax and 0.20 M boric acid bears this out. The results are

plotted in Fig. 1 and show practical identity in the results for the two electrodes as far as they were extended; namely, pH 8.6.

It is necessary to explain the fact that at higher alkalinities the quinhydrone potential is even more positive (oxidizing) than the corrections for factors (Ia) and (Ib) would necessitate. This is brought out most clearly in Table I where the values in Column 6 show a rise to a maximum followed by a decline instead of a continual rise as would be the case if these factors were the only ones operating. It is shown also in Fig. 1 for HCl, where the uncorrected quinhydrone curve (VI) actually crosses the hydrogen electrode curve (IV) at high alkalinity. This rather interesting point, we believe, is cleared up by the experiments of La Mer and Rideal³ in which the rate of autoxidation of hydroquinone at 25°C. was studied as a function of pH by following the absorption of oxygen, the hydroquinone being dissolved in borate buffers. They have found that hydroquinone is apparently inert to the action of molecular oxygen in acid solution, as would be inferred from the fact that stable potentials are obtained for over 25 hours under such conditions with the quinhydrone electrode, but that a measurable oxidation of hydroquinone can be noted at pH 7.3 when vigorously shaken with pure oxygen or at about 7.8 to 8.0 when air is used. The reaction is highly sensitive to further additions of alkali, the rate increasing as the three halves power of the $[H^+]$. The change of hydroquinone into quinone results in an increase of the ratio quinone:hydroquinone with the result that the electrode should drift toward more oxidizing values with time.

The exact pH at which this drift will manifest itself depends not alone upon the pH but also upon the opportunities that are given to facilitate oxygen absorption, as well as the rate at which complicating internal rearrangements of the hydroquinone ions take place.⁴

In one of La Mer and Rideal's experiments it was found that the rate of autoxidation in the presence of air was about 2×10^{-5} gram mols per minute at pH 8.0, the hydroquinone concentration

³ La Mer and Rideal, in press.

⁴ In discussing these results with Professor Bihlmann, we learned that he has recently been successful in applying the electrode for the determination of the pH of soils at alkalinities as high as pH 8.0.

being 0.01 molar. This would correspond at most, since quinhydrone would oxidize more slowly, to an electrode potential change of

$$\Delta \pi = \frac{0.059}{2} \log \frac{0.01002}{0.00998} = 0.00005 \text{ volts per minute}$$

or 0.1 millivolt in 2 minutes. This is just about what we have observed in Table II at pH 8.13. In Table III the drift in potential noted at pH 8.6 was approximately 0.2 millivolt per minute. Our experience with the quinhydrone electrode has shown that it is not necessary to shake the electrode vessel violently to insure equilibrium after each addition of alkali; a slight swirling motion is sufficient to mix the alkaline addition, yielding a prompt equilibrium value. It should accordingly be possible to avoid in large measure the complication of autoxidation for rough titration work by working rapidly (above pH 8.0) and avoiding undue shaking in air. The values so obtained should not be relied upon to give the true pH of the solution for the various reasons enumerated above.

From the discussion given above it seems safe to conclude that the titration values of acids stronger than $K_1 = 10^{-7}$, *i.e.* of the order of monopotassium phosphate, should be reliable. Kolthoff (10) reports some titrations with the quinhydrone electrode designed only to test out how closely the indicator end-point agrees with the quinhydrone end-point. He finds that the two agree to 0.01 cc. in the case of monopotassium phosphate titrated with sodium hydroxide. Owing to the fact that hydroquinone can reduce the alkalinity of alkaline solutions the electrode cannot be used to determine accurately the end-points of weaker acids.

In view of the importance of phosphate buffers at body temperature we have given the result of titrating monopotassium phosphate with sodium hydroxide at 37.5°C. in Table IV. As was to be expected drifting potentials were encountered at lower pH values; namely, 7.61. Another point should be mentioned in this connection. While determining the quinhydrone potentials in acid solution it was noted that a slow drift of a few tenths of a millivolt in observed potentials occurred for the first few minutes after the addition of alkali in the direction of more nega-

tive potentials. A thermometer placed in the titrating vessel showed that the addition of 2 cc. of alkali at 15°C. lowered the temperature of the mixture several degrees and that the change in potential was due solely to readjustment of the temperature to 37.5°C. When correction was made for the temperature immediately after mixing it was found that electrode equilibrium had been obtained within 1 minute. This observation emphasizes the importance of temperature control under such circumstances as the temperature coefficient is greater than one is accustomed to for the hydrogen electrode (0.77 millivolt: degree).

Since the complications, including autoxidation, which beset the use of the quinhydrone electrode in alkaline solution depend primarily upon the extent of the phenolic ionization of the hydroquinone, it would seem that the extension of the method to more alkaline solution can best be brought about by the introduction of a proper substituent in the hydroquinone nucleus so that the degree of phenolic ionization is reduced. So far as we are aware no data exist upon the effect of substitution on the ionization constants of diphenols, but it is reasonable to suppose that negative substituents will increase the acid character, and positive groups, the opposite, as is the case in related systems. Whether the use of a substituted quinhydrone will yield results of practical value in alkaline solution will depend also upon how unfavorably the substituent will affect the system in other directions, such as increasing the reduction potential and rendering the complex quinhydrone less stable, a point which is most essential from practical considerations. We hope shortly to be able to continue this study by correlating the effects of substitution on the reduction potential noted by La Mer and Baker with the change in phenolic ionization and consequent effect on the rate of autoxidation.

SUMMARY.

1. Precise electrometric titrations designed to determine the limits of reliability of the benzoquinhydrone electrode as a means of determining pH have been carried out on hydrochloric, acetic, boric acids, and potassium acid phosphate. Reliable results are obtained in buffered solutions more acid than pH

S.O and the method may be substituted for the hydrogen electrode in such cases, provided no rapid oxidizing or reducing agents are present.

2. The various causes leading to incorrect results in more alkaline solutions are discussed in detail and their relative importance calculated or approximated. Autoxidation of hydroquinone and the effect of the presence of hydroquinone on the actual pH in unbuffered alkaline solutions, owing to its weak acid properties, are more important factors in yielding erroneous results in alkaline solution than are the very small errors which are produced by the deviations from the simple Peters' oxidation-reduction potential equation owing to partial ionization of hydroquinone.

3. The benzoquinhydrone electrode can be substituted for the hydrogen electrode as a more simple and more rapid way of accurately determining the titration end-points of acids (but not bases) that are stronger than the secondary ionization constant of phosphoric acid.⁵

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⁵ In this connection mention should be made of the recent work of Erik Larsson, carried out under the direction of Professor Bjerrum (Larsson, E., *Z. anorg. Chem.*, 1922, exxv, 281) who has found the quinhydrone electrode useful to determine the secondary acid dissociation constants of the following acids, the numbers in parentheses being the values of $K_2 \times 10^6$: succinic (2.4); pyrotartaric (2.3); *l*-malic (7.8); *d*-tartaric (41); fumaric (34); and thiodyglycollic (35). It is likely from his results that the method could be utilized for the determination of the dissociation constants of other sulfur-bearing acids of biological importance, which cannot be obtained by the use of the hydrogen electrode.

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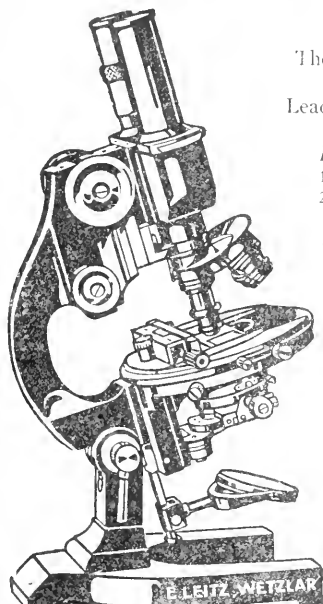
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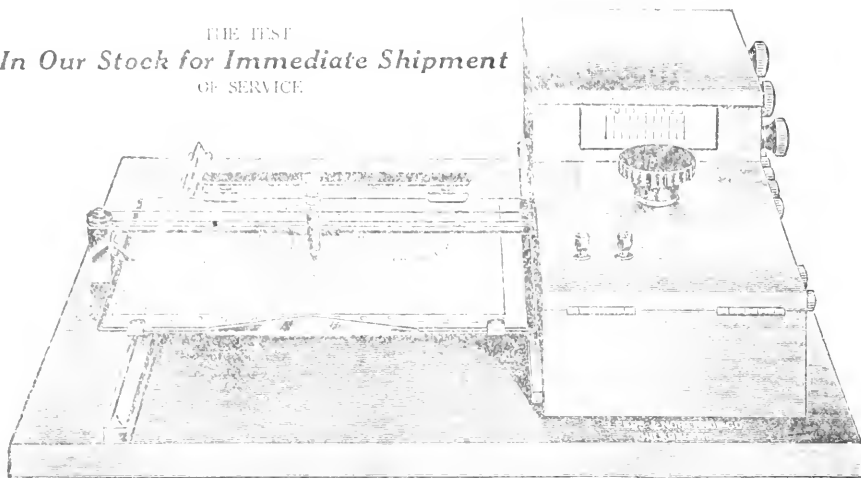
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THE METABOLISM OF FIXED BASE DURING FASTING.*

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(From the Laboratory of the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

(Received for publication, June 30, 1923.)

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I.

INTRODUCTION.

The data presented in this paper were obtained from four epileptic children who were being fasted as a therapeutic measure. It has recently been quite thoroughly shown that epileptic seizures either disappear entirely or else are much reduced as to frequency and severity during the course of a period of fasting, and also that occasionally following a fast there is for a long interval (months) no recurrence of convulsions. The presence of a metabolic factor in the causation of epilepsy is thus definitely indicated. We have at present no knowledge as to whether this factor occupies an initial or a supplementary position in the pathogenesis of the disease. The fact of its presence, however, provides basis for the hope that further study will reveal its

* The expense of this study was in a large part defrayed by a grant from the Epilepsy Medical Research Fund of the Johns Hopkins University.

[†] Cooper Travelling Fellow of McGill University.

character and produce the possibility of devising a rational therapy for this tragic affliction. A considerable amount of investigation has from time to time been undertaken with the purpose of detecting abnormalities of metabolism in epileptics. Very little data of discernible significance have been obtained. The most directly definite finding is that of Jarloev (1). He has demonstrated that preceding epileptic seizures there occurs a measurable shift of the reaction of the blood plasma in the direction of alkalinity. This observation has recently been thoroughly confirmed by Geyelin (2). In connection with this fact may be noted the lowering of the plasma bicarbonate during fasting in consequence of the development of ketosis. This reduction of bicarbonate may be assumed to produce a tendency towards an increased acidity of the plasma, whether or not compensated for by respiratory adjustments. It is therefore possible to regard the beneficial effect of fasting in epilepsy as due to a raised resistance to an alkaline shift in the reaction of the plasma. In any case, the measurements of Jarloev and Geyelin quite definitely indicate an abnormality in epilepsy of the processes concerned in acid-base metabolism, and investigation of the cause of this abnormality should, if successful, greatly increase our knowledge of the pathogenesis of this disease.

Profitable attempt in this direction will require the use of a conception of the manner of acid-base metabolism when the processes concerned are proceeding correctly. We have an excellent knowledge of the principles underlying acid-base metabolism from the point of view of neutrality regulation, due chiefly to the work of L. J. Henderson. It is to be remembered, however, that since acids and bases constitute the chief bulk of substances in solution in the body fluids, their metabolism must be so managed that not only is there sustained in the body water a constancy of reaction, but also a nearly stationary ionic concentration and total ionic mass. In less cumbersome terms the reaction, the osmotic pressure, and the volume of the body fluids are all closely dependent on a correctly controlled metabolism of acids and base. Of the regulatory arrangements sustaining osmotic pressure and volume in the presence of widely variable ratios of acid to base metabolized we are almost entirely uninformed, although certain inferences regarding them may be derived on the basis of the necessary inter-

dependence between them and the known factors concerned in the regulation of reaction.

Assuming the importance of obtaining as accurate a conception as possible of the physiological aspects of acid-base metabolism and of the physicochemical principles underlying its management, it is our purpose in this paper to discuss and illustrate by means of data obtained from fasting children these aspects and principles as far as they are at present visible. We were led to obtain data from fasting epileptics for this illustrative purpose by the reason that the factors concerned in acid-base metabolism are particularly accessible to measurement during fasting. The difficulties in obtaining acid and base measurement from food and feces are avoided. Moreover, there is, owing to the development of ketosis, a large increase in the excess of acid over base claiming excretion from the body fluids, and in consequence operation of the factors sustaining correct acid-base values within the body is excellently exhibited. A further inducement to study acid-base metabolism during fasting is the fact that in consequence of the establishment of an extremely low level of carbohydrate metabolism, there occurs a large reduction of the usual volume of body water, and opportunity is thus provided for obtaining data illustrating the control of acid-base values within the body during a period in which the volume of body water undergoes alteration to a very unusual extent.

In the following presentation and discussion of the data obtained from these children, the evidence, cited above, of a slight maladjustment in epileptics of the processes under consideration, is disregarded. Very possibly the abnormality in question is corrected during fasting. Its persistence, however, would not appreciably interfere with our use of the data obtained as illustrative of the character of the regulatory arrangements which guide the metabolism of acids and base.

II.

Significance of Total Base Concentrations in the Body Fluids.

For the sake of a plan of presentation the regulatory factors in control of acid-base metabolism will be considered chiefly from the point of view of their operation to sustain base concentrations

within the body at constant values. We have chosen to study particularly the manner of maintenance of total base because of its prominence among the values which together sustain the structural integrity of the body fluids. It should be said here, however, that regardless of its relative importance no single structural factor can correctly be expected to be sustained with an absolute rigidity in

the presence of alteration of other factors. The interdependence of the values for the structural factors in blood for example, regarding blood as a physicochemical mechanism, has recently been demonstrated and emphasized by L. J. Henderson (3).

With the purpose of illustrating the importance of the total base value we wish to describe here, with the help of the diagram in Fig. 1, the structural significance of the base concentration in blood plasma. In this diagram the average usual total base concentration, *i.e.* the sum of the separate concentrations of Na^+ , K^+ , Ca^{++} , and Mg^{++} , is represented by the height of the left hand column. Expressed as $0.1 \times$ base this amounts to 156 cc. per 100 cc. of blood plasma. In the right hand column are indicated separately the usual amounts of the acid substances which bind the base of the plasma. Beginning at the top the concentration of

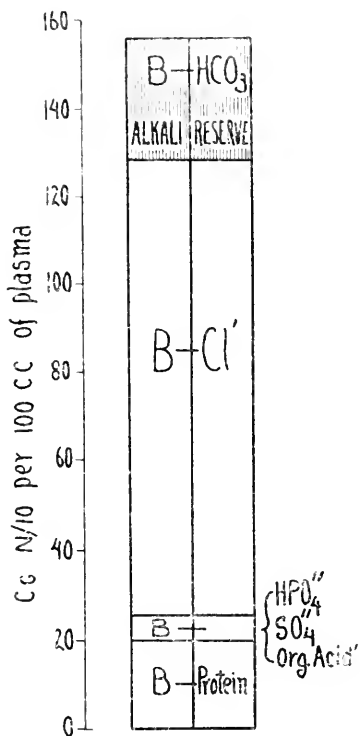


FIG. 1. The normal acid-base composition of blood plasma.

HCO_3^- is seen to bind about one-fifth of the plasma base. Below this value the huge concentration of Cl' covers nearly three-fifths of the base column. The next block in the acid column represents the combined concentrations of HPO_4'' , SO_4'' , and organic acids. It will be seen that after laying off these values a considerable

portion of the base column remains uncovered. It is a reasonable surmise that this remainder of the total base represents the base-binding value of the serum proteins which may, at the reaction of blood plasma, be correctly regarded as acid substances.¹ Four of the acid substances which are carried bound in the plasma are being conveyed for excretion in the urine. These are HCl, H_3PO_4 , H_2SO_4 , and the organic acids. Remembering that a 24 hour urine specimen will usually contain roughly the same amount of each of these four acids, the "threshold" character of the concentration of bound HCl is strikingly apparent in the diagram. The other two values, bound carbonic acid and protein, do not under usual circumstances enter the urine. Remaining in the plasma they have to do with stabilizing the reaction of the blood.

The character of the base in the plasma, *i.e.* the fact that it is practically entirely fixed base, is of cardinal importance from the point of view of the adjustments necessary in the metabolism of acids and base. The minute concentration of the base ammonia in the plasma would be approximately measured by the thickness of the line at the top of the base column. In contrast with this

¹ The values for Na^+ , K^+ , Ca^{++} , and Mg^{++} , used in constructing this diagram are given as mg. per 100 cc. of serum in Table I. Bicarbonate is taken as 60 volumes per cent of CO_2 bound, and chloride as 6.0 gm. of NaCl per liter. The average phosphate content of the serum is assumed to be represented by 5 mg. of P per 100 cc. On the basis of Sørensen's data HPO_4'' at pH 7.4 is assigned 1.8 times its molecular equivalence of univalent base. According to Denis' measurements (4) the plasma contains about 1 mg. of inorganic S per 100 cc. The size of the normal organic acid concentration is not known. It is here given a value of about the magnitude of (HPO_4'') and (SO_4''). The base assumed to be bound by protein is simply the remainder of base after addition of the concentrations taken as normal for the other acid radicles.

The acid-base composition of the plasma in terms of cc. of 0.1 normal per 100 cc., according to these data and as represented in the diagram is as follows:

<i>Base.</i>		<i>Acid.</i>	
Na^+	143.4	HCO_3'	27.0
K^+	5.1	Cl'	103.0
Ca^{++}	5.0	HPO_4''	3.0
Mg^{++}	2.5	SO_4''	1.0
	—	Organic acid	2.0
Total	156.0	Protein	20.0
		Total	156.0

fact is the presence in urine of ammonia to an extent usually of about one-third of the total base excretion. There is thus indicated the fact that the sum of the acid substances derived from a usual diet and claiming excretion in the urine is much in excess of the intake of fixed base, and the necessity for a regulated use of ammonia in the excretion of these acid substances in order that the base concentration of the plasma be sustained is apparent.

The structural importance of the total base value in the plasma can be indicated by reference to the peculiar character of the bicarbonate concentration. The uppermost block in the acid column measuring (HCO_3') may be described as an automatically adjustable value which will immediately change its size following alteration of any of the other values indicated in the diagram. This adjustability of the concentration of bound HCO_3' is a consequence of the fact that carbonic acid is an extremely weak acid and also of the presence in the plasma of an approximately sustained level of free carbonic acid. For the first of these reasons a decrease in total base will be at the expense of bicarbonate or total base remaining stationary; increase in an acid concentration other than (HCO_3') will also reduce bicarbonate. On the other hand, if by any cause the amount of base available for binding HCO_3' be increased, BHCO_3 will at once be formed because of the fact that free carbonic acid is always at hand in the plasma. It follows from these statements that it is the height of the base column which, owing to the elasticity of (HCO_3) , determines the total salt content of the plasma.² The total salt concentration, though not abso-

² In this discussion the adjustability of the acid side of the diagram (Fig. 1) to the base level is attributed entirely to the elasticity of the concentration of HCO_3' . This is practically the case. It should be mentioned, however, that the phosphate and protein values (in terms of base bound) are not to be regarded as immovable in the presence of strong acids claiming base. Secondary phosphates and proteinates, being salts of weak acids, will tend to supply base at the expense of a shift in reaction due to release of acid phosphate and protein. In the case of bicarbonate, however, the tendency to shift in reaction is offset by removal of carbonic acid by way of the lungs. This can be accomplished many times more rapidly than removal of acid phosphate by the kidneys, and protein cannot be removed. It is owing to these facts that (HCO_3') may be assumed to release nearly all of the base needed for neutralization purposes and thus to permit the concentrations of HPO_4'' and protein (in terms of base bound) to remain closely stationary. In other words bicarbonate may be regarded as practically entirely constituting the "alkali reserve."

lutely, is, nevertheless, closely a measure of ionic concentration.³ It is thus evident that a correct osmotic pressure of the plasma is closely dependent on regulation of the process of acid excretion in such manner that the usual base concentration in the plasma is sustained. Obviously, the presence of base in the plasma which can be carried as bicarbonate (the "alkali reserve") is also dependent on the operation of these regulatory factors. The total base value is thus indicated as the underlying structure of correctly built plasma. Upon its maintenance at a closely stationary value will depend the possibility of a usual osmotic pressure and reaction in the plasma.

Presumably, the total base concentration is of the same structural significance in other portions of the body water than blood plasma, and on the basis of osmotic pressure relationships throughout the body fluids it may be assumed to be of approximately the same size in the various compartments of body water. As an illustration of this probability the total base concentration in the water of blood plasma is compared in Table I with that found in the water of human muscle tissue. The data for the four bases in blood plasma are the same as were used in constructing Fig. 1 and are the averages of largely unpublished measurements by Kramer and Tisdall, who have very kindly permitted us to use them here. The water in blood plasma is taken as 91 per cent by volume. The measurements used in computing the base concentrations in muscle water are those of Katz (5) obtained from fresh human muscle. The water content of muscle tissue is taken as 76 per cent by weight. It will be seen that the total base concentrations, in terms of normality, in these examples of extra- and of intracellular water,⁴ are of a similar size. The somewhat higher concentration in muscle water might be explained by reference to the fact that muscle tissue contains two and one-half times as much protein as does blood plasma. The non-dissociable character of proteinates would explain a necessity for a higher base concentration (in terms of normality) in protoplasm in order to provide an ionic concen-

³ Except for the proteinates, the plasma salts are nearly completely dissociable.

⁴ It should be stated that the measurements of Katz include the base contained in the underterminable but presumably relatively small amount of extracellular fluid present in muscle tissue.

tration corresponding to that of extracellular fluids. The few data representing the base content of muscle tissue do not, however, justify surmise beyond the indication that total base is present in roughly the same amount as in blood plasma. A striking fact which may be seen in the table is, that, except for Ca, the concentrations of the individual bases are widely different, Na for instance composing most of the base in plasma water, whereas the greater part of the base contained in muscle water is K. There is thus apparent the fact that the total concentration of base is of service to the organism in a manner quite apart from the local physiological purposes of the concentrations of the individual bases.

TABLE I.

Concentrations of Base in the Water of Blood Plasma and in the Water of Muscle Tissue.

	Per 100 cc. plasma.	Per 100 cc. plasma water.	Per 100 cc. muscle tissue.	Per 100 cc. muscle water.
	mg.	cc. 0.1 N	mg.	cc. 0.1 N
Na ⁺	330	157.7	80	45.8
K ⁺	20	5.6	320	108.0
Ca ⁺⁺	10	5.5	8	5.3
Mg ⁺⁺	3	2.7	21	23.0
Total.....		171.5		182.1

Water of blood plasma is taken as 91 per cent by volume and water of muscle tissue as 76 per cent of weight.

III.

Source of Fixed Base Available for Use in the Process of Acid Excretion during Fasting.

Having indicated the structural importance of the total base concentration in the body fluids it is desired to consider here the extent to which fixed base may be correctly used in the process of acid excretion during fasting. From the fact that there occurs during fasting a continual reduction of the total mass of the body, it may reasonably be inferred that a certain amount of base will enter the urine without causing diminution of base concentrations in the body fluids. The amount of fixed base becoming in this way available for binding acids in the urine cannot be directly

measured, nor may an approximation of its value be arrived at, without a certain amount of conjecture as to its source. The estimations of fixed base availability given below are based on the following assumptions: (1) Except for the greater part of Ca, fixed base within the body may be regarded as in solution in the body water. (2) Fixed base is held throughout the body water at a roughly even level as regards total concentration, the height of this level of content being indicated by the concentration in the water of blood plasma. (3) The consumption of protoplasm will release for use in the process of acid excretion the fixed base contained in the water of destroyed protoplasm. (4) The reduction of the protoplasmic mass during fasting is practically entirely at the expense of the skeletal muscles. Consequently, the availability of base from destroyed protoplasm may be computed with an approximate accuracy from measurements of urine nitrogen and standard values for the amounts of the bases present in muscle water. (5) Owing to a general reduction of the volume of body water, because of the establishment of an extremely low level of carbohydrate metabolism, additional fixed base will, especially during the early part of a fasting period, enter the urine without causing diminution of base concentrations in the body fluids. (6) These premises as regards the source of fixed base and the extent of its availability during fasting may be tested by comparing measurements of fixed base in the urine with amounts estimated as available, using here the further assumption that fixed base excretion is correctly controlled.

Evidence Indicating the Source of the Fixed Base Excretion.—The data given in Table II were obtained from an 8 year old girl who underwent a 15 day fast and was then given during a 3 day after period a light carbohydrate diet consisting of six slices of toast and 75 gm. of cane-sugar per day. The sugar was taken in water flavored with orange juice. This diet was given with the purpose of eliminating the ketone acids as a factor in acid-base metabolism, and it was also expected to raise the level of carbohydrate metabolism sufficiently to permit at least a partial restoration of the usual glycogen content of the tissues. Urine was quantitatively collected during the 18 days of the experiment. The measurements of Na, K, Ca, Mg, nitrogen, and ketone acids given in the table were obtained from six consecutive 3 day speci-

mens. The table also contains calculated values for each of the bases representing the extent of availability from destroyed protoplasm. In obtaining these values the body water released in consequence of the consumption of protein is taken as gm. of urine $N \times 29.5 \times 0.76$, the first factor⁵ providing an estimate of protoplasm destroyed and the second, indicating the corresponding water loss. The amounts of each of the four bases contained in this lost water are computed from the data given in the last column of Table I. The found and calculated values are compared by means of the ratios given in the columns headed F:C.

Looking first in the table at the values for Ca, it will be at once apparent that enormously more Ca is excreted during fasting than could possibly be derived from destroyed protoplasm. For the whole period of the fast, ten times as much Ca is found in the urine as may be calculated as available from muscle tissue consumption. Obviously there is other source of Ca. Presumably there occurs a resolution of Ca from deposits in osseous tissue. This inference is supported by the finding of Goto (6) that the calcium carbonate content of the bones of rabbits is reduced following feeding with mineral acids. As might be expected it is the more soluble salt, carbonate rather than phosphate, which reenters the body fluids. This salt, moreover, better serves the base need since its acid radicle is disposed of base-free by way of the lungs.

Considering next the values for K and Mg, it will be noted that again in both instances more base is found in the urine than is calculable on the basis of urine N. The amounts found for the whole fasting period are, however, only moderately in excess of the amounts calculated as available; F:C being 1.3 for K and 1.4 for Mg. Looking at the values for the individual periods of the experiment it will be noted that the greatest excess of found over calculated occurs during the first period, and that during the last three periods of the fast the F:C ratios are very close to unity. Explanation is provided by the fact that there occurs at the outset of fasting a large reduction of the volume of body apart from the water lost in consequence of destruction of protoplasm. This fact is made evident by the much more rapid loss of weight during the

⁵ Taking N in muscle tissue as 3.4 per cent, $100 \div 3.4 = 29.5$.

TABLE II.

Measurements of Fixed Base Excretion during a 15 Day Fast and a 3 Day Carbohydrate After Period of A.C., and Amounts of Base Calculated from Measurements of Urine N as Available from Destroyed Protoplasm.

	Period (3 days)	Nitro- gen.	Na*		K*		Mg**		Ca**		Ketone acids (as β - oxybuty- ric),	Plasma protein.				
			Found.	Calcu- lated.	F:C*	Found.	Calcu- lated.	F:C	Found.	Calcu- lated.			F:C			
Fasting.	I	gm.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	gm.	percent				
	II	21.3	783	219	3.6	897	515	1.7	195	110	1.8	115	23	5.0	14.9	8.1
	III	16.1	293	165	1.8	512	389	1.3	135	83	1.6	224	18	12.5	23.6	7.8
	IV	13.3	42	137	0.3	283	322	0.9	77	69	1.1	202	15	13.4	17.5	8.6
	V	11.5	77	118	0.7	286	278	1.0	57	59	1.0	135	13	10.4	13.0	8.1
Total.....		10.3	61	106	0.6	277	249	1.1	48	53	0.9	124	11	11.3	10.3	7.9
		72.5	1,256	745	1.7	2,255	1,753	1.3	512	374	1.4	800	80	10.0	79.3	
Carbohydrate.	VI	7.6	16	78	0.2	67	184	0.4	17	39	0.4	56	8	7.0	1.4	7.4

* F:C represents the ratio of found to calculated.

early part of a fast than subsequently. In the case of this subject, for instance, the weight lost was 1.46 kilos during the first 2 days of the fast as compared with 0.13 kilo during the last 2. This reduction of the water content of the tissues is apparently referable to the establishment of an extremely low level of carbohydrate metabolism; glucose, after consumption of the body's slender store of surplus glycogen, being available only to the extent to which it is produced by the metabolism of protein. Biscoff and Voit (7) and Benedict and Milner (8) have demonstrated that when a diet containing carbohydrate abundantly is replaced by one consisting of fat and protein only, which is in effect the case during fasting, there occurs a large loss of body water. The inverse aspect of this phenomenon may be illustrated by the fact that in the case of this subject during the 3 day carbohydrate after period, although the food intake was far short of the energy requirement, there occurred a gain in body weight amounting to 0.7 kilo. The nature of the effect of the level of carbohydrate metabolism on the volume of body water is obscure. Presumably it is a physical effect depending on the concentration of glycogen in body water. Possibly it is also in relationship with the amount of circulating glucose. The extra loss of weight during the early part of a fast is certainly many times greater than the weight of the consumed surplus of glycogen. The water lost is therefore not to be explained to an appreciable extent as molecular water of glycogen. Water from this source, moreover, would not be accompanied by an excretion of constituents of body water. The excretion of K and Mg, during the first two periods of this subject's fast, in excess of amounts estimated as from the water of destroyed protoplasm, may therefore reasonably be regarded as a consequence of reduction of the volume of tissue water, the released water carrying with it into the urine its content of these bases. During the three remaining periods of the fast K and Mg are apparently derived only from the water of consumed protoplasm. In agreement with the conception which is being used the excretion of K and Mg during the carbohydrate after period is very greatly reduced, the F:C values being in both instances 0.4. This may be explained as indicating a retention of K and Mg, derived from destroyed protoplasm, with the purpose of sustaining their concentrations in an expanding volume of body water.

To note next the values found for the remaining base Na, it will be seen that the excess of found over calculated on the basis of destroyed protoplasm as its source is, during the first period of the fast, much greater than in the case of K and Mg. This larger excess can be explained by using the fact that Na is carried in the extracellular body water at a concentration several times greater than in cell water (see Table I). Shrinkage of this compartment of body water will obviously throw a quantity of Na into the urine in addition to that derived from the water of destroyed protoplasm and from decrease in cell volume. Assuming that the reduction of the volume of body water in consequence of the lowered level of carbohydrate metabolism is not confined to intracellular water, the F:C ratio for Na is, on the basis of the above considerations, correctly higher than the ratios for K and Mg. That a reduction of the volume of extracellular water does occur during fasting is fairly satisfactorily indicated by the high values found for serum protein which are placed in Table II as evidence on this point. These measurements were taken at the end of each 3 day period. The values during the fast may be compared with the concentration found at the end of the carbohydrate after period which is close to the average usual value. It will be noted in the table that the F:C ratios for Na fall, after the second period, below unity. If the calculated values for available Na be taken as dependable the inference is permissible that, during the latter part of the fast, there occurs to some extent replacement of the loss of extracellular water which occurred during the first two periods. As regards the cause of the reduction of extracellular water, it is reasonable to expect that the loss of intracellular water due to glycogen consumption will be accompanied by alteration of the volume of extracellular water. Presumably the two values are closely interdependent. Data to be presently presented (Table V) suggest that the loss of extracellular water follows reduction of cell volume.

Estimations of Body Water Loss Based on Measurements of N, K, and Na in the Urine.—As may be seen in Table I, K is the chief base in muscle water and Na constitutes nearly all the base in plasma water. If it be permissible to take the values given in Table I as representative of the concentrations of K and Na in other portions of intra- and extracellular water, respectively,

estimations may be obtained from measurements of K and of Na in the urine of the loss of body water from these two compartments. Estimations of the water loss during fasting derived on this basis are presented here. They will be compared presently with estimations of the water loss obtained in another manner. The concentration of K in extracellular water is a relatively insignificant value and the loss of extracellular water is, moreover, presumably a much smaller quantity than the loss of intracellular water. For calculation purposes K in the urine from extracellular water is neglected and all the K is regarded as derived from the loss of intracellular water. The total K excretion for the 15 day fast of A.G. was 2,255 cc. of 0.1 N (Table II). The K content of 1 cc. of muscle water is 1.08 cc. of 0.1 N (Table I). The loss of intracellular water during the fast of A.G. may therefore be taken as $2,255 \div 1.08 = 2,090$ cc. This loss is due to two causes: a destruction of protoplasm and a reduction of cell volume. In Table II, K presenting for excretion along with the water of destroyed protoplasm, calculated on the basis of urine N, is given as 1,753 cc. of 0.1 N. The water which contained it is $1,753 \div 1.08 = 1,620$ cc. If this value for water loss from destruction of protoplasm be subtracted from the total loss of intracellular water, 2,090 cc., the remainder, 470 cc., may be taken as the water loss due to reduction of cell volume.⁶

To obtain an estimate of the loss of extracellular water, Na calculated as from the water of destroyed protoplasm, 745 cc. of 0.1 N (see Table II), is subtracted from the total Na in the urine,

⁶ The following calculation is presented as a rough attempt to obtain some idea of the magnitude of the alteration of cell volume caused by reduction of what may be called, among the factors determining cell volume, the "glycogen factor." The 470 cc. of water lost by reduction of cell volume is here taken as wholly derived from muscle tissue. The weight of A.G. before the fast was 24.7 kilos. Taking 40 per cent of the body weight as muscle tissue, 9.88 kilos are obtained. Urine N. 72.5 gm. \times 29.5, indicates a loss of 2.14 kilos. The muscle mass, assuming the "glycogen factor" intact, is thus reduced by the end of the fast to 7.74 kilos. The additional loss of 470 cc. of water, due to reduction of the "glycogen factor," therefore represents a 6 per cent ($470/7,740$) decrease in cell volume. This is doubtless an overestimation, for the reason that the loss of cell water is probably not confined to muscle cells. On the other hand, the full size of the "glycogen factor" is not measured since glycogen is not by any means entirely removed from the tissue cells during fasting.

1,256 cc. of 0.1 N, and the remainder, 511 cc. of 0.1 N, is regarded as having accompanied the loss of extracellular water. The Na content of serum water per cc. is given in Table I as 1.58 cc. of 0.1 N. The extracellular water lost during the fast is thus indicated as $511 \div 1.58 = 320$ cc.

On the basis of these estimations the loss of body water during the 15 day fast of A.G. may be stated as follows:

Intracellular water lost.

1. Due to destruction of protoplasm 1,620 cc.
2. " " reduction of cell volume 470 "

2,090 "

Extracellular water lost 320 "

Total loss of body water 2,410 "

Loss of body weight 3,920 gm.

Body weight loss due to water 62 per cent.

In Table III are presented measurements of urine N, Na, and K, from another subject, M.C., a 12 year old girl who underwent a 10 day fast. The analyses were obtained from urine collections covering periods of different lengths and the values are for this reason given per 24 hours. It may be seen that these data are of quite the same character as those obtained from A.G. (Table II). From the measurements of urine N, Na, and K, for the entire 10 day fast the following statements are derived by the plan of calculation used above.

Intracellular water lost.

1. Due to destruction of protoplasm 1,400 cc.
2. " " reduction of cell volume 700 "

2,100 "

Extracellular water lost 470 "

Total loss of body water 2,570 "

Loss of body weight 3,820 gm.

Body weight loss due to water 67 per cent.

In comparing the estimations of water loss for these two children it should be noted that M.C. underwent a fast which was

in duration two-thirds of the fasting period of A.G. and that she was a much larger child, weighing at the beginning of her fast 38.4 kilos, the initial weight of A.G. being 24.7 kilos. The fact of the shorter fast of M.C. may be used to explain the relatively larger size, among the values obtained for this subject, of the loss of water due to reduction of cell volume, this change being practically entirely effected during the early part of a fasting period. The values for percentage of water in the weight loss, 62 and 67 per cent, arrived at in this round about way are of a quite likely size. A lower percentage of water than obtains in muscle tissue (76 per cent) would be expected because of the presence in the weight loss of a considerable amount of fat. The quite reasonable

TABLE III.

Measurements of Na and K Excreted in the Urine during the 10 Day Fast of M.C., and Amounts of These Bases Calculated from Measurements of Urine N as Available from Destroyed Protoplasm.

Days of experiment.	Ketone acids (as β -oxybutyric).	Nitrogen.	Na			K		
			Found.	Calculated.	F:C	Found.	Calculated.	F:C
	gm.	gm.	cc. 0.1 N	cc. 0.1 N		cc. 0.1 N	cc. 0.1 N	
I		5.60	101	57	1.8	362	135	2.7
II, III, IV	6.97	6.97	253	72	3.5	331	169	2.0
V, VI, VII, VIII	8.47	6.12	108	63	1.7	181	148	1.2
IX, X	6.64	5.98	92	61	1.1	60	145	0.4

character of these estimations of body water loss tends to confirm the premises which are being used regarding the source of fixed base becoming available for use in the process of acid excretion during fasting. They also indicate a usefulness of measurements of Na and K in the urine as a means of studying alterations in the volume of body water.

Estimations of Water Loss Based on Measurements of Nitrogen and Ketone Acids in the Urine and of the Loss in Body Weight.—With the purpose of roughly testing the values for water loss arrived at from measurements of N, Na, and K in the urine, the total loss of body water is here computed in a different manner; viz., by subtracting a calculated value for the weight of fat and protein consumed from a measurement of the loss of body weight. The weight of the surplus glycogen consumed is neglected. The

amount of protein burned is obtained by multiplying the grams of urine N by the usual factor, 6.25. Body fat which was completely oxidized is computed from urine N by means of Shaffer's recently published values for ketogenic and antiketogenic equivalents (9). The incompletely oxidized fat is calculated from a measurement of the ketone acid excretion. Shaffer's values stated in a form convenient for our purpose are as follows:

	Ketogenic.	Antiketogenic.*	Excess.
1 gm. fat =	3.43 millimols.	1.14 millimols.	2.29 millimols ketogenic.
1 gm. urine N =	15 “	40 “	25 millimols anti- ketogenic.

* As triose.

A molecule of triose being taken as the measure of carbohydrate required for the complete oxidation of a molecule of fatty acid, it may be at once derived from the equivalents here given that 1 gm. of urine N indicates the complete oxidation of $25 \div 2.29 = 10.9$ gm. of fat. That fat has been used to this extent and beyond is evident from the presence of ketone acids in the urine. To obtain a factor by means of which to calculate the weight of incompletely oxidized fat from measurement of the ketone acids (as β -oxybutyric); if 1 gm. of fat = 2.29 excess ketogenic millimols, or $2.29 \times 104 = 238$ mg. of β -oxybutyric acid, then 1 gm. of β -oxybutyric acid may be taken to indicate the incomplete oxidation of $1,000 \div 238 = 4.2$ gm. of fat.

Using these factors the calculation of the weights of fat and of protein oxidized, and, as a matter of incidental interest, of the energy expenditure, during the fasts which these two girls underwent can be briefly presented.

Case 1.—A.G., 8 years old, 15 day fast.

Nitrogen in urine.....	72.5 gm.
Ketone acids in urine (as β -oxybutyric).....	79.3 “
Average weight during fast.....	22.7 kilos.
$72.5 \times 6.25 =$	453 gm. protein oxidized.
$72.5 \times 10.9 =$	790 “ fat oxidized completely.
$79.3 \times 4.2 =$	333 “ “ “ incompletely.

1,576 “ body protein and fat consumed.

Energy Expenditure.

Protein, 453 × 4.1	= 1,860 cal.
Fat, 1,123 × 9.3	= 10,440 "
	<hr/>
	12,300 "
Lost in β -oxybutyric, 79.3 × 4.5	= 360 "
	<hr/>
Total energy used	11,940 "
Energy per 24 hrs., 11,910 ÷ 15	= 796 "
" " 24 " per kilo, 796 ÷ 22.7	= 35 "

Case 2.—M.C., 12 years old, 10 day fast.

Nitrogen in urine	61.8 gm.
Ketone acids in urine (as β -oxybutyric)	69.1 "
Average weight during fast	36.5 kilos.
61.8 × 6.25 = 386 gm. protein oxidized.	
61.8 × 10.9 = 674 " fat oxidized completely.	
68.1 × 4.2 = 286 " " " incompletely.	

1,346 " body protein and fat consumed.

Energy Expenditure.

Protein, 386 × 4.1	= 1,580 cal.
Fat, 960 × 9.3	= 8,920 "
	<hr/>
	10,500 "
Lost in β -oxybutyric, 68.1 × 4.5	310 "
	<hr/>
Total energy used	10,190 "
Energy per 24 hrs., 10,190 ÷ 10	= 1,019 "
" " 24 " per kilo, 1,019 ÷ 36.5 =	28 "

The value for energy expenditure arrived at in this way may be regarded as a reasonable estimate in the case of A.G. The 28 calories per kilo, per 24 hours, calculated for M.C. is doubtless too low, although she was an older and extremely quiet child. The fact that these calculations of energy expenditure based on two measurements in the urine are evidently near the mark must be taken as highly commendatory of Shaffer's values, and as indicating that the weight of fat consumed computed in this way is a satisfactorily approximate value.

The measurements of the loss of body water which these data provide may now be presented and compared with the values derived on the basis of the excretion of N, Na, and K.

	A.G.	M.C.
Body weight loss	3,920	3,820
Protein and fat oxidized	1,576	1,346
Body water lost	2,344	2,474
“ “ “ calculated from N, Na, and K in urine.	2,410	2,570

These estimations of body water loss from measurements of weight loss, and of nitrogen and ketone acids in the urine are seen to be in satisfactory agreement with those derived from measurements of urine N, Na, and K.

Fixed Base Availability during Fasting. Computed on the Basis of the Estimated Water Loss and the Normal Total Base Concentration in the Water of Blood Plasma.—The values for loss of body water obtained by subtracting the calculated weight of fat and protein consumed from the loss of body weight are here multiplied by the average usual concentration of base in the water of blood plasma. This value is given in Table I as 1.72 cc. of 0.1 N per cc. of plasma water and is taken as representing the level of base content throughout the body water. The amounts of fixed base calculated in this way as available for use in the process of acid excretion during the fasting periods of A.G. and M.C. are compared with the measurements of base in the urine. For the purposes of this comparison, however, it is necessary to deduct an amount of Ca assumed to be derived from Ca deposits. An estimation of this value can only be arrived at by using again the calculated loss of water and its Ca content, obtained on the basis of the value given for Ca in plasma water in Table I. The Ca in this way calculated as from body water is then subtracted from the total Ca in the urine in order to indicate Ca derived from a source other than body water. These data are as follows:

A.G., 15 day fast.

Body water lost	2,344 cc.
Calculated available fixed base. .	$2,344 \times 1.72 = 4,030$ “ of 0.1 N.
Fixed base found in urine (Table II).	
Na ⁺	1,256 cc. of 0.1 N.
K ⁺	2,255 “ “ 0.1 “
Mg ⁺⁺	512 “ “ 0.1 “
Ca ⁺⁺	800 “ “ 0.1 “

Total 4,823 cc.

Calculated Ca available from body water, $2,344 \times 0.055 = 129$ cc. of 0.1 N.

Ca in urine from Ca deposits, $800 - 129 = 671$

Total fixed base in urine from body water, $4,823 - 671 = 4,152$ cc. of 0.1 N.

M.C., 10 day fast.

Body water lost 2,474 cc.

Calculated available fixed base $2,474 \times 1.72 = 4,260$ cc. of 0.1 N.

Fixed base found in urine.

Na⁺ 1,378 cc. of 0.1 N.

K⁺ 2,262 " " 0.1 "

Mg⁺⁺ 556 " " 0.1 "

Ca⁺⁺ 654 " " 0.1 "

Total 4,850

Calculated Ca available from body water, $2,474 \times 0.055 = 136$ cc. of 0.1 N.

Ca in urine from Ca deposits, $654 - 136 = 518$ cc. of 0.1 N.

Total fixed base in urine from body water, $4,850 - 518 = 4,332$ cc. of 0.1 N.

The calculated values for available fixed base during the fasts of these two children are thus, considering the certainly only approximate accuracy of the factors used, in excellent agreement with the amounts of fixed base found in the urine. These data satisfactorily support the conception that, except for most of the Ca, the fixed base used in the process of acid excretion during fasting is the base content of the lost body water. The inference is also obtained that fixed base, except for Ca, is used only to the extent of its availability from this source. These data are obviously not of a precision to permit the statement that no fixed base was excreted at the expense of base concentrations within the body. The approximate agreement, however, of base found with base calculated as available clearly indicates the control of fixed base excretion in defense of these concentrations. Evidence more directly indicating the accuracy of this control will be presented in a subsequent section.

Further Illustration of the Factors of Body Water Excretion during Fasting.—In appreciation of the regulatory ability of the mechanism sustaining base in body water it is to be noted that during fasting there occurs not only a very unusual excess of acid over base claiming excretion in the urine, but also considerable

alteration in the volume of body water. The character of the body water loss during the course of the 15 day fast of A.G. is illustrated by the data in Table IV and is repeated graphically in Fig. 2. The several values which account for the loss of body weight are here roughly evaluated for each of the five 3 day periods of the fast. The measurements used, Na, K, N, and ketone acids (as β -oxybutyric), are those already given in Table II. The manner of calculation of the separate values with the help of the additional data in Table I has been described above. That they are of an accuracy quite sufficient for their illustrative purpose may be indicated by noting that the total body weight loss during the 15 day fast obtained by adding together all the calculated values for the five periods is 3,986 gm., comparing satisfactorily with the loss of 3,920 gm. found by weighing the subject before and at the end of the fasting period. The diagram (Fig. 2) may therefore be regarded as displaying in approximately correct perspective the factors accounting for the loss in body weight during the course of the 15 day fast. As regards the water loss, it will be seen that it is largest during the first period of the fast, owing chiefly to the fact that the lowering of the level of the water content of the body due to reduction of the "glycogen factor" is in a large part effected during the first 3 days of fasting. After 6 days of fasting the loss of water from this cause has practically ceased. Apparently the loss of extracellular water is also limited to the first two periods of the fast. This point cannot, however, be regarded as dependably demonstrated by the data at hand. The chief purpose of the diagram is to indicate that, owing to both an absolute and a relative decrease in body water, base concentrations within the body will require constant adjustment if they are to be kept at usual values. A fact of incidental interest appearing in the table and to be seen also in the diagram is the roughly constant ratio of protein to fat used for energy purposes. From the point of view of the size of the ketone acid production, it is significant that body protein is used to an extent proportionate to the amount of fat oxidized with the result that the degree of ketosis is defined. The actual extent of the need of protein for maintenance purposes is presumably indicated by the nitrogen excretion during the carbohydrate after period which, as may be seen in Table II, is about one-third of the urine N during the first period of the fast. It is

perhaps permissible to interpret the so called "protein sparing effect" of ingested carbohydrate as simply a removal of the need of sugar obtained from protein as a means of limiting ketone acid production.

TABLE IV.

Factors of Weight Loss of A. G. during a 15 Day Fast Calculated from Measurements of N, Ketone Acids (as β -Oxybutyric), Na, and K in Urine Given in Table II.

Period (3 day).	I	II	III	IV	V
	gm.	gm.	gm.	gm.	gm.
Protein oxidized	133	100	83	72	64
Fat completely oxidized	232	176	145	125	112
" incompletely oxidized	63	99	74	55	43
Extracellular water lost	357	81	-60*	-26	-28
Intracellular water lost.					
1. Due to reduction of cell volume . .	353	114	-36	8	26
2. " " destruction of protoplasm.	477	360	298	257	230
Total weight loss†	1,615	930	504	490	447
Gm. protein \div gm. fat	0.45	0.36	0.38	0.40	0.41

* The minus sign indicates a calculated gain in water.

† The sum of the calculated values for weight loss is 3,986 gm. The weight loss directly measured was 3,920 gm.

Data similar to those just presented, illustrating the composition of the weight loss of Subject M.C. during her 10 day fast, are contained in Table V. The values there given are per 24 hours for the reason that they were obtained from urine collections covering irregular divisions of the fast; *viz.*, the first day, the next 3 days, the following 4, and the last 2 days. The decline in the loss of extracellular water, and of intracellular water due to reduction of the "glycogen factor," during the course of the fast is again shown. Apparently both of these values are to a slight extent replenished during the last 2 days of the fast at the expense of water from destruction of protoplasm. The data, however, are scarcely of an accuracy to establish this inference. An additional point of interest appearing in this table is indicated by the values found for the 1st day of the fast; *viz.*, that the loss of intracellular water seems to begin before there is evidence of appreciable loss from the extracellular fluids.

It is emphatically desired that the calculated values given in this section be regarded as not more than extremely rough approximations. As regards, for instance, the use of the K content of muscle water as a means of estimating the loss of intracellular water during fasting, it is admitted that water is quite certainly

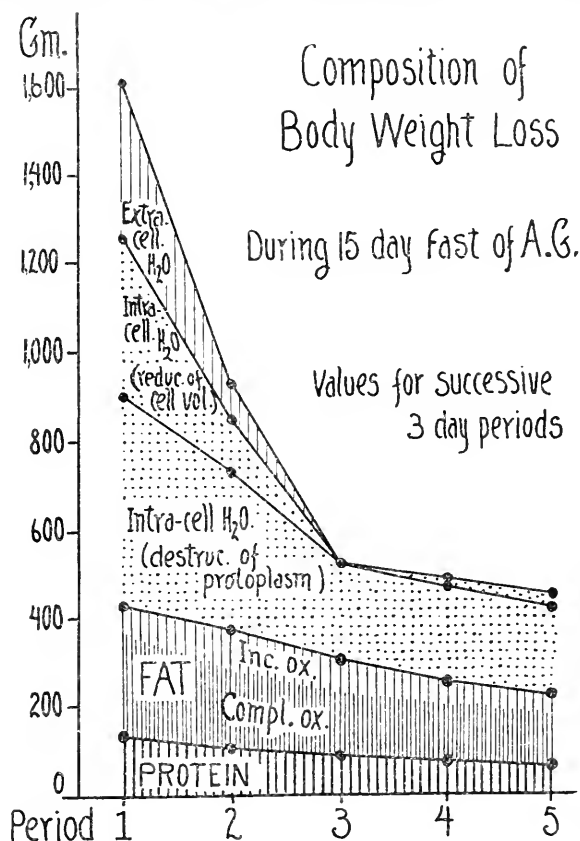


FIG. 2. Constructed from data in Table IV.

lost from other tissues than the skeletal muscles. The assumption that this plan of calculation will provide a rough approximation of the intracellular water loss depends on the probability that the skeletal muscles provide nearly all of the protein used during fasting, and also on the possibility, indicated for instance by the K content of

red blood cells, that other portions of intracellular water contain concentrations of K of somewhere near the size found in muscle water. It may also be noted here that in referring the availability of inorganic base to "destruction of protoplasm," it is not intended to convey the conception that muscle tissue is consumed en bloc. The alternative view, a general assessment of all of the cells for protein for the energy need, would seem to be much more reasonable. A choice of conjecture is, however, unnecessary in using the

TABLE V.

Factors of Weight Loss of M.C. during a 10 Day Fast Calculated from Measurements of N, Ketone Acids (as β -Oxybutyric), Na, and K in Urine, Given in Table IV.

Days of experiment.	I	II, III, IV	V, VI, VII, VIII	IX, X
	gm.	gm.	gm.	gm.
Protein oxidized.....	35	44	38	37
Fat completely oxidized.....		76	67	65
“ incompletely oxidized.....		29	35	30
Extracellular water lost.....	28	115	45	20
Intracellular water lost.				
1. Due to reduction of cell volume....	210	150	31	-55*
2. “ “ destruction of protoplasm..	125	156	137	79
Total weight loss†.....		570	353	176
Gm. fat ÷ gm. protein.....		0.42	0.37	0.39

* The minus sign indicates a calculated gain in water.

† The calculated weight loss for the last 9 days of the fast is on the basis of these data 3.47 kilos. The weight loss directly measured was 3.55 kilos.

conception that with reduction of protoplasm there occurs a proportionate release of its largest constituent, water.

It is our impression that the data given are of an accuracy sufficient to permit the inference that all of the inorganic base appearing in the urine during fasting, except for the greater part of the Ca excretion, may with probable correctness be regarded as the base content of body water lost in consequence of causes referable to the metabolism of fasting. The source of the base is, we believe, thus adequately explained without the necessity of hypotheating the existence in the body of "base depots," except in the case of Ca. The data are presented with the purpose of

illustrating in terms sufficiently quantitative this extremely simple conception of the factors determining the availability of inorganic base for the binding of acid in the urine during fasting.

IV.

*Factors Determining the Amounts of Acid Substances
Claiming Excretion in the Urine during Fasting.*

The outstanding feature of the energy metabolism during fasting is that it very soon comes to be practically entirely supported by the combustion of body fat and protein, with the result that oxidation of carbohydrate is reduced to a level determined by the extent to which glucose is derived from the metabolism of body protein. The amounts of the various acid substances claiming excretion in the urine during fasting are in relationship with this lowering of glucose availability. The most conspicuous consequence is an incomplete oxidation of a considerable part of the body fat used in support of the energy need. The incompletely oxidized fatty acids must be excreted in the urine as β -oxybutyric and diacetic acids with an expenditure of nearly their full equivalence of base. Since under normal circumstances the final acid product of the oxidation of the fatty acids, H_2CO_3 , is eliminated base-free by way of the lungs, the ketone acids produced during fasting constitute a large addition to the usual excess of acid over fixed base claiming excretion in the urine and make necessary great extension of the factors conserving the base concentrations of the body fluids. The extent to which the oxidation of fat is incomplete during fasting is illustrated by the data in Tables IV and V and is also shown in Fig. 2.

Besides the incomplete oxidation of a part of the body fat used during fasting, which results in a large addition to the acid excretion, there occurs also an oxidation of body protein in excess of the maintenance need of the vitally important parts of the protoplasmic mass and a reduction of the water content of the body in excess of the water loss accompanying the destruction of protoplasm. Both of these additional effects of a lowered glucose availability are concerned in determining the amounts of acid substances derived from protein and body water which will enter

the urine during fasting. Proportionate to the destruction of protoplasm, amounts of H_2PO_4 , H_2SO_4 , HCl , and presumably of the organic acids found normally in the urine, will be released and will claim excretion, and to such extent as these acid substances are present as inorganic salts in the body water there should occur an additional excretion of them proportionate to the loss of body water in excess of the water of destroyed protoplasm. HCl being carried altogether as chloride and, moreover, being contained chiefly in extracellular water, its excretion during fasting may be expected to be largely a consequence of the decrease in body water volume due to reduction of the "glycogen factor." As this event occurs during the first part of a fasting period the excretion of chlorides should be most abundant then, declining subsequently to a small amount representing the chloride content of the water of destroyed protoplasm, the concentration there (muscle water) being according to Katz one-fifth of that found in extracellular water (plasma water). The basis for this expectation regarding the character of the chloride excretion during fasting will be made apparent by inspection of Fig. 2, and satisfactory verification is provided by the measurements of HCl excretion during the 15 day fast of Subject A.G. given in Table VI. In complete contrast to HCl , H_2SO_4 is practically entirely derived by oxidation of protein. The amounts of sulfates in the urine may therefore be expected to parallel closely the consumption of protoplasm regardless of alterations in the volume of body water. In agreement with this anticipation are the approximately stationary ratios for urine N and S (inorganic) given in Table VI. Katz notes that half or even more of the phosphorus contained in muscle tissue may be extracted with water. This fact provides ground for the surmise that the water of protoplasm may contain a considerable concentration of inorganic phosphates. If this be the case a part of the excretion of phosphates during fasting should be a consequence of the reduction of cell volume, and the urine during the early part of a fasting period should therefore contain P in addition to the amount derived from the destruction of protoplasm. The declining character of the P:N ratio in Table VI, except for a high ratio during Period IV, may be interpreted in support of this surmise. The very low ratio for the carbohydrate after period may be taken, in agreement with the conception

which is being used, as indicating a retention of P to support the phosphate concentration in the presence of an expanding cell volume due to restoration of the "glycogen factor." The high P:N ratio during the first few days of fasting in contrast with the very low ratio when glucose is given, and also the roughly stationary

TABLE VI.

Measurements of Nitrogen, Sulfates, Phosphates, and Chlorides in Urine during the 15 Day Fast and 3 Day Carbohydrate After Period of A.G.

	Period (3 day).	Nitrogen.	Sulfates (inor- ganic).	S: N	Phos- phates (in- organic).	P: N	Chlorides.
		<i>gm.</i>	<i>gm. S</i>		<i>gm. P</i>		<i>gm. Cl</i>
Fasting.	I	21.3	1.26	0.058	2.35	0.110	1.56
	II	16.1	0.94	0.058	1.67	0.104	0.80
	III	13.3	0.77	0.058	1.17	0.088	0.60
	IV	11.5	0.57	0.050	1.10	0.096	0.26
	V	10.3	0.57	0.055	0.89	0.086	0.14
Carbohydrate.	VI	7.6	0.42	0.056	0.29	0.038	0.14

TABLE VII.

Measurements of Nitrogen, Sulfates, and Phosphates in Urine during the 4 Day Fast and 2 Day Carbohydrate After Period of D.M.

	Day.	Nitrogen.	Sulfates (inorganic).	S: N	Phos- phates (in- organic).	P: N
		<i>gm.</i>	<i>gm. S</i>		<i>gm. P</i>	
Fasting.	I	5.74	0.336	0.058	0.605	1.05
	II	6.73	0.472	0.062	0.718	0.94
	III	7.56	0.428	0.057	0.800	1.05
	IV	8.12	0.433	0.053	0.832	1.02
Carbohydrate.	I	5.53	0.280	0.051	0.468	0.85
	II	5.18	0.259	0.050	0.220	0.42

value of the S:N ratio in the presence of a change in cell volume are illustrated again by the data in Table VII. The measurements used here were obtained from an eight year old boy (Subject D.M.) who was fasted for 4 days and then given cane-sugar and toast for 2 days. The decline of the P:N ratio during the course of a fasting period is here interpreted in the same way as the much larger change in the found:calculated ratio for K given in Table II.

The wider range of the latter ratio may be explained as due to the fact that K is entirely contained in cell water and its excretion may, therefore, be expected to be affected by alteration of cell volume to a greater extent than in the case of the P of protoplasm which is present only in part in solution as phosphates.

v.

The Regulation of Fixed Base Excretion.

The amounts of fixed base found in the urine collected during the fasting periods of A.G. and M.C. are in approximate agreement with the amounts calculated as available for use in the process of acid excretion without depleting the base content of body water (Section III). The extent to which fixed base enters the urine is thus seen to be carefully controlled. In this section will be presented data illustrating the manner of this control. The necessity for a constantly alert regulation of fixed base excretion is evident from the fact that, even under usual circumstances of metabolism, the sum of acid substances entering the urine is variably larger than the intake of fixed base. The huge increase in the excess of acid over fixed base claiming excretion in the urine during fasting, owing to the causes considered in Section IV, obviously demands a wide extensibility of the factors effecting the conservation of fixed base if the base concentrations of the body fluids are to be correctly sustained. As regards the manner of the control of fixed base excretion, we are chiefly indebted to Henderson (10) and to Henderson and Palmer (11) for an essentially complete understanding of the principles underlying the operation of this very beautiful example of a physiological mechanism of the regulatory type. In undertaking to describe briefly this mechanism, the two factors which, together, determine the extent to which the acid products of metabolism are permitted to carry fixed base into the urine may be designated "base economy" and "ammonia production."

The base economy factor depends on the ability of the kidney to secrete a fluid of much greater acidity than blood plasma. In consequence of this fact phosphoric acid and various organic acids can enter the urine with less base than they bind while being con-

veyed for excretion, and carbonic acid is almost entirely prevented from carrying base into acid urines.

The extent of the absolute saving of base in the elimination of phosphoric acid in urine of usual acidity (pH 6.0) may be briefly indicated. Phosphoric acid is bound both as mono-basic and as di-basic phosphate in the plasma and in urine. In the plasma (at pH 7.4) the ratio $\text{BH}_2\text{PO}_4:\text{B}_2\text{HPO}_4$ is 1:4. In urine (at pH 6.0) the ratio is more than reversed, becoming 9:1. From these ratios it may be derived that the base bound (in terms of normality) by H_3PO_4 while being conveyed for excretion is 1.8 times the molecular concentration of the acid, whereas in urine at pH 6.0 the base equivalence is reduced to 1.1. Thus, according to these values, the elimination of phosphoric acid in urine of usual acidity is accomplished with a saving of approximately 40 per cent of the base which it binds in the plasma. A further slight saving is obtainable by the secretion of urine of maximum acidity, pH 5.0.

The various organic acids which normally enter the urine are presumably weakly acid substances and it may reasonably be expected that in acid urines a part of their total concentrations will be present unbound. That these acids are to an appreciable extent free in acid urines may be demonstrated by comparing the titratable acidity of the urine with the amount of acid phosphate present. The former value is found to exceed to a definitely measurable extent the acidity due to acid phosphate. In the case of the ketone acids knowledge of their dissociation constants (12) permits us to state that in very acid urines containing them, about 5 per cent of the diacetic acid and 20 per cent of the β -oxybutyric acid are unbound. With the very large excretion of these acids which takes place during fasting, the economy of base in their elimination, although small in relation to their total excretion, nevertheless becomes a value of about the same absolute size as the saving effected in the case of phosphoric acid.

The elimination of carbonic acid in urine is the result of processes which differ essentially from those which determine the excretion of the other urinary acids. Free carbonic acid is not actively secreted by the kidney, its presence in the urine being simply a direct diffusion consequence of the CO_2 tension of the plasma. It may therefore be regarded as only incidentally a

urinary acid. The concentration of H_2CO_3 in the urine, being determined by that of the plasma, is an approximately stationary value, direct measurements indicating a slightly higher concentration than obtains in the plasma.⁷ Owing to this fixed value for free carbonic acid the concentration of bicarbonate in the urine necessarily becomes a function of hydrogen ion concentration, and declines rapidly with increase in urinary acidity.⁸ The significance of these relationships may best be indicated by actual values. Urine excreted at the reaction of blood plasma, pH 7.4, is found to contain about 360 cc. of 0.1 N BHCO_3 per liter, approximately the bicarbonate concentration of the plasma, whereas in urine of average acidity (pH 6.0) the concentration of BHCO_3 is reduced to 10 to 15 cc. of 0.1 N per liter. Inasmuch as carbonic acid can be eliminated base-free by way of the lungs, bicarbonate entering the urine must be regarded, from the point of view of base conservation in the process of acid excretion, as base entirely wasted. It is evident from the data just given that by elimination of urine of a usual degree of acidity a large and entirely useless expenditure of base is almost completely avoided. This fact must be regarded as a chief significance of the usual reaction of urine. The factors controlling the elimination of carbonic acid in urine are thus seen to suit excellently the usual need for an economical use of base in the process of acid excretion. It should be pointed out, however, that these factors fit just as appropriately the inverse of the usual situation; *viz.*, an excess of fixed base over acid claiming excretion in the urine. Under these circumstances depression of urinary acidity will permit the entrance into the urine of increasingly large amounts of fixed base as bicarbonate. By this manœuvre depletion of acid concentrations in the body fluids, (Cl') for instance, can be avoided. The use of carbonic acid in the process of acid excretion in defence of other acid concentrations within the

⁷ In explanation of this somewhat higher CO_2 tension in the urine, it may be surmised that the urine acquires the CO_2 tension of the kidney tissue and that this is somewhat above, though in direct relationship with, the CO_2 tension of the plasma.

⁸ Hydrogen ion concentration being the quotient of the $\frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3}$ ratio, it necessarily follows that, (H_2CO_3) remaining stationary, (BHCO_3) will decline with increase in (H').

body is directly analogous to the excretion of ammonia as a means of sparing fixed base. These two substances are correctly comparable in that they present in common a widely extensible availability. This point is made in order to indicate that both sides of the acid-base structure in the body water are strongly defensible. The above description of the factors controlling the excretion of base in the urine as bicarbonate is derived from data published by Gamble (13).

The two remaining urinary acids, hydrochloric and sulfuric, being strong acids, *i.e.* dissociating when in solution practically all of their hydrogen, cannot be present to an appreciable extent unbound in so weakly acid a solution as is urine of even maximum acidity. They must therefore carry their full equivalence of base into the urine.

With regard to measurement of the base economy factor, it may first be noted that the small amount of carbonic acid which enters the urine as a consequence of the CO_2 tension of the plasma is almost entirely lost during and after voiding unless elaborate precautions are taken in collecting the urine. The acidity of a urine specimen collected in the usual way is therefore practically entirely due to acid phosphate and free organic acids. The base saving which this acidity represents can, as Henderson pointed out, be easily and directly measured by titrating the urine to the reaction of blood plasma (pH 7.4), the amount of alkali which must be added to urine to bring its reaction to that of blood plasma being obviously a measure of the base released by these acids as they entered the urine. The measurements of base economy given below were obtained in this manner from 24 hour urine specimens collected without precautions against a loss of CO_2 , and may therefore be taken as representing the base saving in the elimination of phosphoric acid and the organic acids. The huge saving of base as bicarbonate which is a consequence of the excretion of acid urine (pH 6.0 to 5.0) has been described above. Urines obtained during fasting are nearly always of a pH below 6.0 and therefore contain negligible amounts of base as bicarbonate. For this reason measurements of H_2CO_3 and BHCO_3 are not included in the data given below which in other respects present completely the acid-base composition of the urine. These data are meant to illustrate the manner of excretion of the strictly urinary acids

among which carbonic acid is appropriately not included. It should not be forgotten, however, that owing to the ability of the kidney to secrete these acid urines management of the excretion of the acids which must leave the body in the urine is unhampered by a large wastage of base as bicarbonate.

The second factor controlling fixed base excretion consists in a regulated use of ammonia for the binding of acid substances as they enter the urine. As regards the latter part of this statement we have not direct evidence. We see, however, in the urine often the greater part of an abnormally large acid excretion bound by NH_3 , and find in the blood plasma a concentration of NH_3 which is so small as to be not accurately measurable (± 1.0 cc. of $0.1 \times$ per liter). It is easy to assume from these facts that the transport of acid substances is accomplished by means of fixed base and that this base is replaced in the kidney by NH_3 , derived from urea, to such extent as is necessary for the maintenance of the fixed base concentration of the plasma. Such an hypothesis involves crediting the kidney with accomplishment of a regulated synthesis, a rôle which it must be admitted seems startlingly out of character for this organ. Nash and Benedict (14), however, have recently advanced argument and experimental evidence which strongly support the surmise that the kidney is the locus of the regulatory relationship between ammonia production and acid excretion. As compared with base economy it may be noted that ammonia production is usually the larger of the two factors controlling fixed base excretion and in time of need is greatly more extensible.

The data to be presented in this section were obtained from three children. (1) A. G., 8 years old, whose 15 day fast and 3 day carbohydrate after period have already been mentioned (Section III). The measurements given here are 24 hour values for consecutive 3 day periods during the 18 days of the experiment. (2) F. MeH. was a 12 year old girl. For 3 days before her fast she was placed on an adequate standard diet consisting of bread 100 gm., butter 20 gm., and milk 800 cc. After fasting for 10 days, this diet was resumed for a period of 1 week, and was quantitatively taken, including the 1st day that her fast was broken. Beginning the day before the fast 24 hour urine specimens were collected throughout the entire experimental period. (3) D. M., an 8 year old boy, was also placed on a standard diet,⁹ in this instance for 5 days before his fast. He fasted 4

⁹ The diet of D.M. during the fore period was per day: milk 1,000 cc., bread 100 gm., butter 20 gm., farina 200 gm., one egg, and one orange.

days and was then given carbohydrate only for a period of 5 days. This after period diet consisted of four slices of toast and 50 gm. of cane-sugar per day. The sugar was given in water flavored with orange juice. During the last 3 days of the fore period and throughout the fast and after period, 24 hour urine specimens were collected. Measurements from only the two first specimens following the fast are given here. During the last 3 days of the after period NaCl was added to the carbohydrate diet for a purpose to be described in the next section. These children were given distilled water to drink. The water was kept at their bedsides and they were encouraged to drink as much as they wished to.

With the purpose of illustrating completely the process of acid excretion the following substances were directly determined in the urine specimens: organic acids, phosphates (inorganic), sulfates (inorganic), chlorides, titratable acidity, and ammonia. From these measurements, acid and base values, as cc. of 0.1 N, were derived. The acid values are given in terms of base equivalence at the reaction of blood plasma, *i.e.* H_3PO_4 as cc. of 0.1 M, was multiplied by 1.8 (see foot-note 13), and the full titration value of the organic acids as measured by the method of Van Slyke and Palmer was taken. As has been mentioned, HCl and H_2SO_4 are completely bound both in urine and in plasma. By subtracting base economy (titratable acidity) from the total acid excretion¹⁰ (in terms of base bound at pH 7.4) a measurement of the total base excretion is obtained. If the measurement of ammonia excretion be subtracted from the value for total base excretion the remainder of base may be taken as the amount of fixed base which was permitted to enter the urine. It will be seen that by this plan an indirect measurement of the fixed base excretion is obtained, and the operation of the two factors controlling it is incidentally illustrated in quantitative terms. This method of analysis of the process of acid excretion is shown in detail by means of actual data in Table VIII. The indirectly obtained measurement of the fixed base excretion is necessarily

¹⁰ It should be noted that, in this paper, by the term "total acid excretion" is meant, as indicated above, the sum of the measurements of the acid radicles in urine. This meaning of the term should therefore not be confused with that of "total acid" used by Henderson and Palmer to designate the sum of the measurements of the titratable acidity of the urine and the ammonia excretion; *i.e.*, the amount of acid excreted without an expenditure of fixed base.

TABLE VIII.
Analysis of the Process of Acid Excretion (Data from Urine Collected during the 4 Day Fast of D.M.).

I. *Indirect estimation of fixed base excretion.*

Acid excretion in terms of base bound at pH 7.4.

Organic acids; directly titrated	= 2,400 cc. 0.1 N organic acids.
Phosphates; $2,955 \text{ mg. P} \div 3.1 \times 1.8$	= 1,715 " 0.1 " H_3PO_4 .
Sulfates; $1,669 \text{ " S} \div 3.2 \times 2$	= 1,045 " 0.1 " H_2SO_4 .
Chlorides; $2,942 \text{ " Cl} \div 3.5$	= 840 " 0.1 " HCl .

6,000 " 0.1 " total acid excretion.
= 1,226 " 0.1 " base economy.

Titratable acidity of urine

4,774 " 0.1 " total base excretion.
= 1,701 " 0.1 " ammonia production.

Ammonia; $2,890 \text{ mg. NH}_3 \div 1.7$

3,073 " 0.1 " fixed base excretion.
--

II. *Fixed base excretion directly measured.*

Na, mg. $3,073 \div 2.3$	= 1,336 cc. 0.1 N.
K, " $5,320 \div 3.9$	= 1,362 " 0.1 "
Ca, " $266 \times 2 \div 4.0$	= 133 " 0.1 "
Mg, " $312 \times 2 \div 2.3$	= 260 " 0.1 "
<hr/>	
	3,091 " 0.1 "

of only an approximate accuracy. Table VIII contains also a direct measurement of the fixed base obtained by determining separately the amounts of Na, K, Ca, and Mg in the specimen, and it will be seen that the total of fixed base found is, in this instance, in excellent agreement with the indirect estimation of fixed base excretion. Further evidence that the principles used in obtaining the fixed base excretion in this manner are correct is supplied by the data given in Table IX. It will be noted that only in a few instances is there a wide discrepancy between the calculated and directly measured values.

TABLE IX.

Comparing the Direct Measurements and Indirectly Obtained Estimations of Fixed Base Excretion during the 15 Day Fast and the 3 Day Carbohydrate After Period of A.G.

	Measured directly.	Estimated indirectly.
	cc. 0.1 N	cc. 0.1 N
1st 3 days of fast.	663	652
2nd 3 " " "	388	332
3rd 3 " " "	201	194
4th 3 " " "	185	195
5th 3 " " "	170	177
Carbohydrate, 3 days.	52	14

Values given are per 24 hours.

The data obtained from the three children are presented in Tables X, XI, and XII, according to the plan indicated in Table VIII. Measurements of pH, nitrogen, creatinine, and of the A:NH₃, and NH₃ nitrogen : total nitrogen ratios are also included in these tables. The values in the tables are repeated graphically in Figs. 3, 4, and 5. In these figures the left hand diagram represents the acid excretion and the diagram on the right the manner of its management as regards the use of base. The values for the individual acids are superimposed with the result that the top line measures the total acid excretion. The right hand diagram is constructed by first copying the line representing the total acid excretion (in terms of base bound at the reaction of the plasma), then laying off downward from it the measurements of titratable acidity (base economy). There is thus produced a line measur-

TABLE X.

Analysis of the Process of Acid Excretion during a 15 Day Fast and a 3 Day Carbohydrate After-Period. Data from A.G.

	Period* (3 day).	Volume.	pH	Acid excretion.†				Base economy (titratable acidity).	Total base excre- tion.	NH ₃	Fixed base.	A NH ₃	NH ₃ N Total N.	Creati- nine.
				Organic acids.	H ₃ PO ₄	H ₂ SO ₄	HCl	Total.						
Fasting.	I	cc. 817	5.3	cc. 0.1 N 675	cc. 0.1 N 455	cc. 0.1 N 264	cc. 0.1 N 149	cc. 0.1 N 1,543	cc. 0.1 N 1,254	cc. 0.1 N 602	cc. 0.1 N 652	0.48	per cent 12	mg. 335
	II	1,050	5.6	857	324	196	77	1,454	1,244	912	332	0.23	24	293
	III	1,017	5.9	627	225	162	57	1,071	952	758	194	0.16	24	287
	IV	1,047	6.0	463	212	120	25	820	722	530	192	0.18	20	247
	V	807	6.0	403	173	120	14	710	623	446	177	0.20	18	241
Carbohydrate.	VI	800	6.6	131	56	87	11	288	250	236	14	0.25	13	238

* Values given are per 24 hours.

† In terms of base bound in plasma (*i.e.* at pH 7.4).

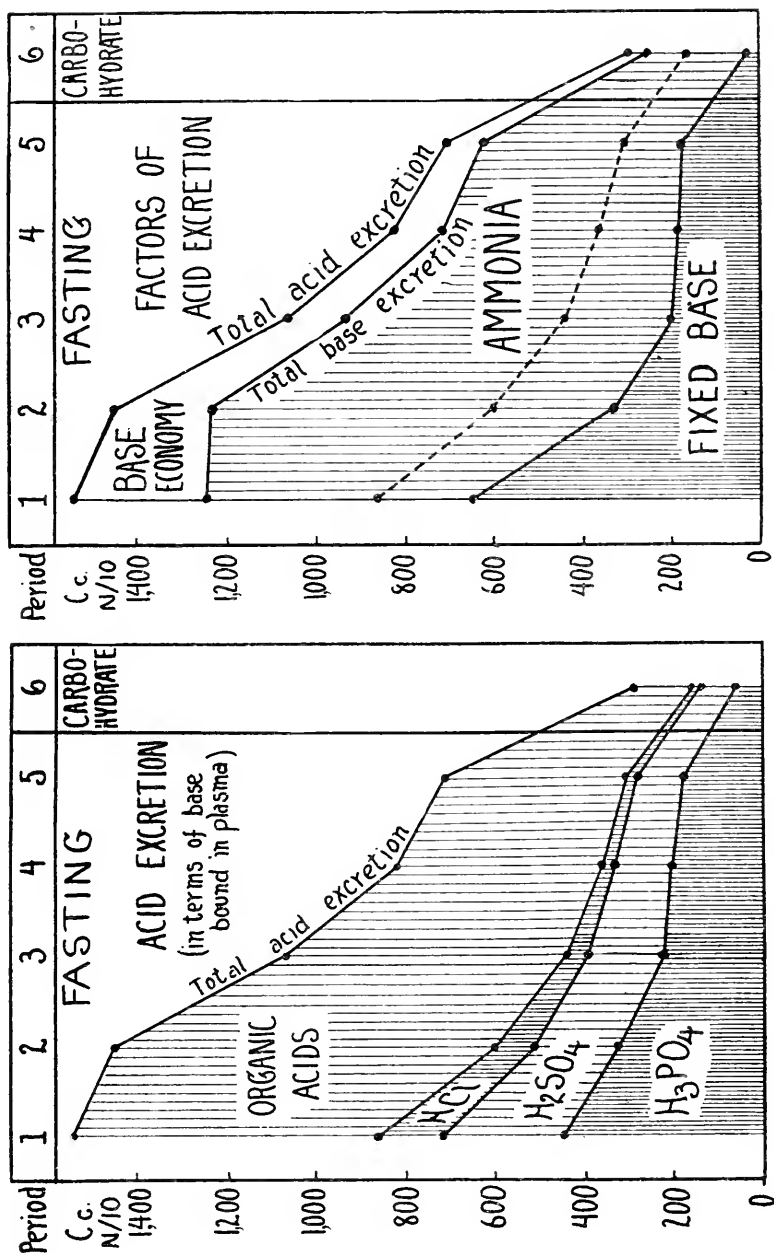


FIG. 3. Presenting graphically the data from A. G. given in Table X.

TABLE XI.

Analysis of the Process of Acid Excretion during a 10 Day Fast and during a Fore and After Period on a Standard Diet. Data from F. Mell.

	Day of experiment.	Vol- ume.	pH	Acid excretion.*					Base economy (titratable acidity).	Total base excretion.	NH ₃	Fixed base.	A NH ₃	NH ₄ N Total N†	Creatinine.
				Organic acids.	H ₂ PO ₄	H ₂ SO ₄	HCl	Total.							
		cc.		cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N		per cent	mg.
Fore period.	Average of 3 days.	680	6.2	166	362	248	562	1,308	148	1,160	194	966	0.76	4	542
Fasting.	I	600	6.0	178	281	155	383	997	123	874	141	733	0.87	4	474
	II	300	5.2	570	396	214	112	1,292	252	1,040	256	794	0.98	6	490
	III	500	5.1	898	397	186	36	1,517	353	1,164	483	681	0.73	11	454
	IV	510	5.2	891	318	186	62	1,457	318	1,139	621	518	0.51	14	450
	V, VI†	630	5.3	1,009	311	180	72	1,572	276	1,296	840	456	0.33	20	479
	VII	480	5.5	968	266	172	60	1,466	221	1,245	913	332	0.19	26	516
	VIII	440	5.5	908	222	161	67	1,358	186	1,172	857	315	0.22	26	523
	IX+														
	X	370	6.2	488	199	126	50	863	106	757	625	132	0.17	19	491
After period.	I and II†	320	6.5	176	140	166	38	520	55	465	318	147	0.17	9	483
	III	240	6.5	149	108	142	64	463	49	414	316	98	0.16	9	478
	IV	390	5.9	160	133	122	292	707	76	631	322	309	0.24	9	431
	V	550	6.1	141	196	143	362	842	89	753	202	551	0.44	5	451
	VI	700	6.6	157	232	144	476	1,009	68	941	168	773	0.40	4	459
	VII	720	6.9	186	276	176	562	1,200	50	1,150	148	1,002	0.34	4	538

* In terms of base bound in plasma (i.e. at pH 7.4).

† 24 hour values from 48 hour specimens because of error in timing collections.

‡ Specimen incomplete.

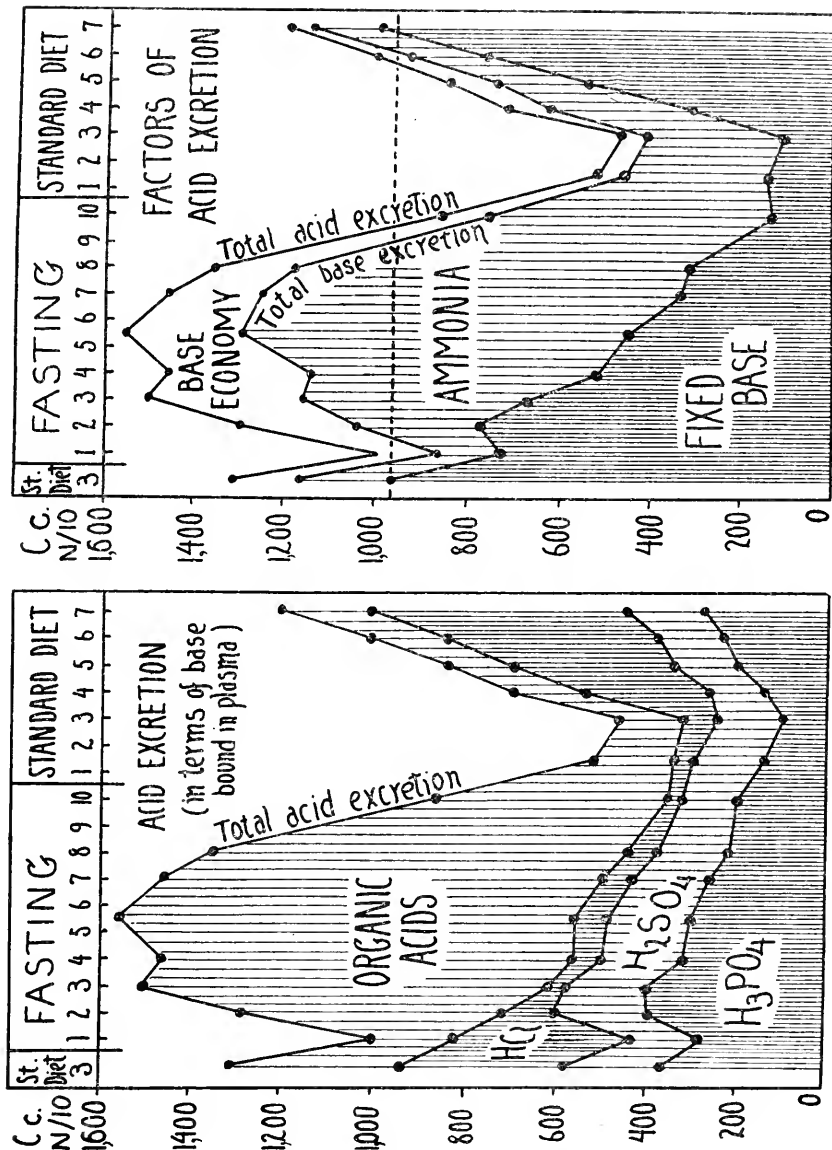


Fig. 4. Presenting graphically the data from F. McH. given in Table XI.

TABLE XII.

Analysis of the Process of Acid Excretion during a Fast Period on a Standard Diet, a 4 Day Fasting Period, and a 2 Day Carbohydrate After Period. Data from D.M.

	Day of experiment.	Vol- ume.	pH	Acid excretion.*				Base economy (titratable acidity).	Total base excretion.	NH ₃	Fixed base.	A. NH ₃ .	NH ₃ N Total N [†]	Creatinine.
				Organic acids.	H ₂ PO ₄	H ₂ SO ₄	HCl	Total.						
Fast period. Standard diet.		cc.		cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N			mg.
	Average of 3 days.	880	5.2	146	383	287	637	1,453	288	1,225	973	0.91	5	325
Fasting.	I	650	5.2	154	351	211	329	1,045	209	836	675	1.30	4	345
	II	660	5.0	589	417	297	168	1,471	326	1,145	875	1.21	5	395
	III	640	5.1	820	461	269	196	1,749	373	1,376	862	0.73	10	331
	IV	510	5.4	837	483	272	147	1,739	318	1,421	665	0.42	13	334
Carbohydrate.	I	370	5.6	484	272	176	106	1,038	160	878	326	0.29	14	250
	II	520	6.6	144	128	163	191	626	46	580	160	0.11	11	348

* In terms of base bound in plasma (i.e. at pH 7.4).

ing the total base excretion. From this line the amounts of ammonia found in the urine are laid off and the size of the remainder of the base excretion, which is fixed base, is thus indicated.

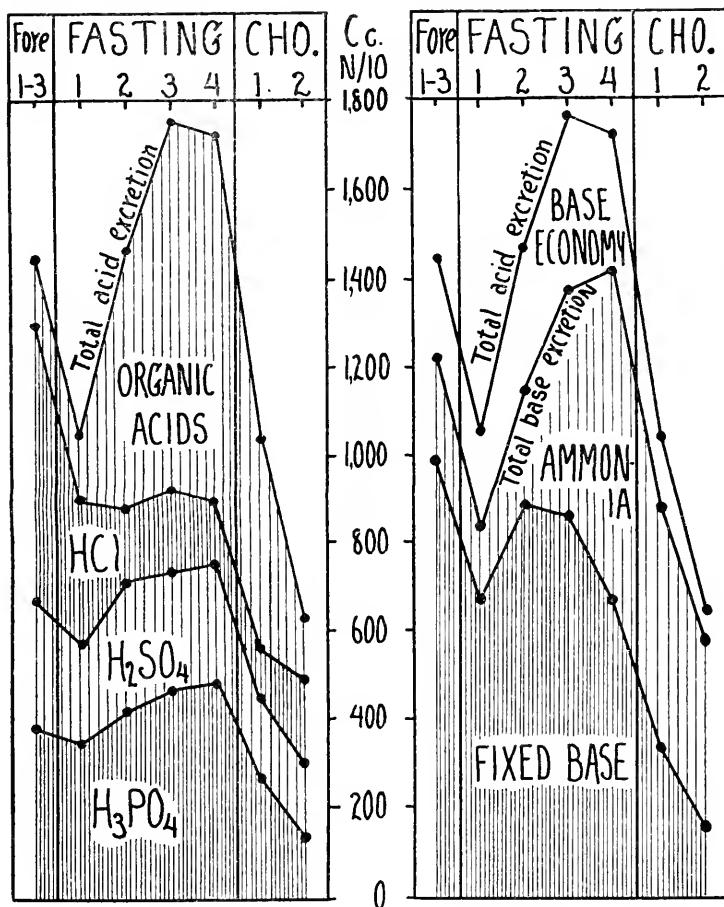


FIG. 5. Presenting graphically the data from D. M. given in Table XII.

It will be obvious that these lines are not true curves, since they simply connect points measuring 24 hour values which are vertically superimposed.

These data are presented with the purpose of indicating the character of the acid excretion during fasting and the manner of its accomplishment. This purpose, it is believed, is satisfactorily served by the plan used in presenting the values in the tables and figures, and further comment may be brief. The huge increase in the organic acid excretion, due to the ketosis of starvation, is evident in the tables and diagrams. For instance, in terms of the total acid excretion the organic acids were, in the case of F. MeH., 13 per cent on the fore period diet, and became 64 per cent at the height of ketosis (5th and 6th day of the fast), (Table XI and Fig. 4). These values for D.M. are 10 and 50 per cent on the fore period diet, and on the 4th day of fasting, respectively, (Table XII, Fig. 5).¹¹ The organic acid excretion is seen to rise

¹¹ It is of interest to note here the several times larger organic acid excretion which would take place were it not that the ketosis is limited by combustion of protein in excess of the maintenance need of the vitally important parts of the protoplasmic mass (see page 653). In illustration of this point, urine N was in the case of D.M. 8.12 for the 4th day of his fast. Urine N for the 5th day of the carbohydrate period following the 4 day fast was found to be only 2.38 gm. Assuming that these 2.38 gm. of N represent the maintenance need for protein, then $8.12 - 2.38 = 5.74$ gm. of N may, for the purpose of this argument, be taken as protein metabolized for the sake of its yield of triose. Were this extra protein not used the fat incompletely metabolized in consequence would be, according to the factors given on page 649, $10.9 \times 5.74 = 63$ gm., and the ketone acid excretion therefrom, as β -oxybutyric, $63 \div 4.2 = 15$ gm. The excretion of ketone acids during the 4th day of D.M.'s fast was, as β -oxybutyric, 5.2 gm.; i.e., one-fourth of the amount which would have claimed excretion if protein metabolism had been limited to the maintenance need.

It may be calculated that management of the excretion of an additional 15 gm. of ketone acids would be, in this instance, far beyond the capacity of the mechanism controlling fixed base elimination. Assuming that 20 per cent of these approximately 1,500 cc. of 0.1N ketone acids would enter the urine unbound 1,200 cc. would have to be covered by NH_3 . The NH_3 excretion on the 4th day of the fast was 750 cc. of 0.1 N. To manage the excretion of the additional ketone acids a total of 1,950 cc. of 0.1 N NH_3 would be demanded which in terms of ammonia N would be 2.73 gm., an amount in excess of the total urine N (2.38 gm.) taken as indicating the use of protein to the extent of the maintenance need. These data will serve to emphasize the great importance from the point of view of acid-base metabolism during fasting of the antiketogenic rôle of protein metabolism. As has been mentioned, the so called "protein sparing effect of carbohydrate" may probably be regarded as the removal of a demand for antiketogenic substance at the expense of protein.

rapidly during the first 4 or 5 days of fasting, presumably in consequence of the consumption of the body's surplus of glycogen, and then to fall steadily during the remainder of the fasting period. It is also to be noted that decline in organic acid excretion is accompanied by a decline in the excretion of the other urinary acids. Indeed after the height of the organic acid excretion has been reached there obtains throughout the remainder of the fast a quite strikingly steady proportionality as regards the amounts of organic and of inorganic acids entering the urine. To illustrate this point the organic acid:total acid ratios derived from the data in Table X for Periods II, III, IV, and V, are 0.59, 0.58, 0.56, and 0.57, respectively. This relationship may be satisfactorily explained. During the first several days of fasting the body's store of surplus carbohydrate is rapidly consumed and the decrease in the volume of body water due to reduction of the "glycogen factor" is almost completely effected. During the remainder of the fasting period the extent to which protoplasm is destroyed practically entirely determines the inorganic acid excretion and also, owing to the stationary ratio of body fat and body protein consumption (Tables IV and V), determines the organic acid excretion. It may therefore be expected that, with the gradual lowering of energy metabolism during fasting, the amounts of inorganic and of organic acids entering the urine will decline in the parallel fashion which may be seen in Table III and as indicated by the ratios given above. It may be mentioned here that inference that the height of ketosis during fasting represents a temporarily excessive use of fat over protein is incorrect, the grade of ketosis being determined simply by the extent of energy expenditure which is sustained, after the disappearance of surplus glycogen, at the expense of body fat and body protein on a steady basis of partnership.

Tables X, XI, and XII, and especially Figs. 3, 4, and 5, illustrate clearly the limitation of the use of fixed base in the process of acid excretion effected by the two regulatory factors, base economy and ammonia production. In the case of F. McH., for instance, (Table XI and Fig. 4) on the fore period diet, 70 per cent of the total acid excretion is bound by fixed base, whereas on the 10th day of the fast fixed base is used to the extent of only 15 per cent of the acid claiming excretion. The fixed base excretion is seen

to decline extensively during the course of the fasting periods. In agreement with the considerations which have been presented regarding the factors which determine the availability of fixed base during fasting, this decline is well shown in Fig. 3 to be at first rapid and then gradual. The much larger amounts of fixed base entering the urine during the first, and, to less degree, during the second, period of A.G.'s fast than subsequently illustrate effectively the release of base, in addition to that derived from destruction of protoplasm, due to the general reduction of the volume of body water which is almost completely effected during the early part of the fast. During the third, fourth, and fifth periods of the fast the base being derived only from destroyed protoplasm, the amounts entering the urine diminish gradually with reduction of the extent of protein consumption accompanying the slow decline in energy expenditure. The effect of restoring the "glycogen factor" is strikingly shown by the almost complete disappearance of fixed base from the urine during the carbohydrate after period, fixed base from destroyed protoplasm being evidently retained to uphold base concentrations in a rising level of body water. These points have been already presented. They are mentioned here again because they are so well illustrated in Fig. 3. The control of fixed base excretion in order to sustain base concentrations during reestablishment of the usual volume of body water is also excellently illustrated by the after period values in the experiment with F. MeH. Here (Table XI and Fig. 4), fixed base intake being the same as during the fore period, its excretion is seen to rise gradually, not reaching its fore period value (indicated by the dotted line across the diagram) until the 7th day of the after period.

It may be noted here again that the fixed base excretion and the inorganic acid excretion are both determined by the extent to which protoplasm is destroyed and the volume of body water reduced during fasting. To illustrate this relationship, the points measuring the total inorganic acid excretion of A.G. in Fig. 3 have been transferred to the diagram on the right and connected by a dotted line, which it will be seen parallels fairly closely the line measuring fixed base excretion. Its position above the latter is explained by the fact that in the destruction of protoplasm the release of H_2SO_4 and H_3PO_4 from protein, produces acid in excess of

that bound by fixed base in the cell water. This excess and the total organic acid excretion constitute the value which must be covered by the two factors controlling fixed base excretion.

As regards the cooperation of these factors, it may be noted that the capacity of the base economy factor is not extensible beyond the value which can be obtained by secreting urine at the physiological limit of acidity. The increase in the capacity of this factor obtained by secreting urine at pH 5.0 instead of at the usual average reaction, pH 6.0, is in terms of the total acid excretion, relatively very small.¹² Ammonia being presumably derived from the neutral end-product of protein metabolism urea, a wide extension of its usual value would seem to be possible; urea N constituting under average conditions as regards diet about 70 per cent and ammonia N 5 to 10 per cent of the total urine N. Under usual circumstances of metabolism the ammonia production factor is but slightly larger than base economy, the average value for the ratio of the titratable acidity of the urine and the ammonia excretion being, according to Henderson and Palmer (11), 0.80. The values given in Tables X, XI, and XII for the A:NH₃ ratio and also for the "ammonia coefficient" (ammonia N:total N) will satisfactorily demonstrate that during fasting the control of fixed base excretion is chiefly accomplished by a very unusual extension of the ammonia production factor. In Table XI for instance it may be seen that the A:NH₃ ratio falls from its fore period value of 0.76 to 0.17 and that the "ammonia coefficient" rises correspondingly from 4 to 26 per cent. It is to be noted, however, that the base economy factor is apparently the more alert of the two, extending rapidly to its full capacity as indicated by urinary pH, during the first several days of fasting. This point is also illustrated by a rise of the A:NH₃ ratio above unity during

¹² The base equivalence of phosphoric acid at the reaction of blood plasma, pH 7.4, is 1.8; at the usual reaction of urine, pH 6.0, it is 1.12; and in acid urine, pH 5.0, it is 1.02. For derivation of these data see foot-note 13. From them it may be estimated that the base saving in the elimination of phosphoric acid in urine of pH 6.0 is 38 per cent of the base bound by HPO₄" in the plasma and that in urine of pH 5.0 this saving is increased only to 43 per cent.

In the case of the ketone acids, however, the greater part of the possible base economy (10 to 20 per cent of their equivalence) is obtained by secretion of urine below pH 6.0.

TABLE XIII.
Partition of the Base Economy Factor from Data from F. McIL.

Period.....	Fore.		Fasting.										After.					
	3		1	2	3	4	5-6	7	8	9	10	1-2	3	4	5	6	7	
Day.....	3		106	178	173	136	134	111	94		68	38	29	58	71	54	38	
Acid phosphate,* cc. 0.1 N.....	124		17	74	180	184	142	110	92		38	17	20	18	18	11	12	
Free organic acid, cc. 0.1 N.....	24																	
Organic acid: acid phosphate.....	0.18		0.16	0.42	1.04	1.35	1.06	0.99	0.98		0.56	0.45	0.69	0.31	0.25	0.26	0.32	

* In excess of acid phosphate at pH 7.4.

the first 2 days of the fast of D. M. (see Table XII). Subsequently it will be noted (see Tables X and XI) that there occurs a considerable variation of pH with a tendency to return to the average usual value in urine; pH 6.0. These facts indicate that the bulk of the base saving is a result of the greatly increased ammonia production and suggest that the final adjustment of fixed base excretion is accomplished by elimination of acid and alkaline phosphate in appropriate ratio. Obviously, the 1:1 ratio

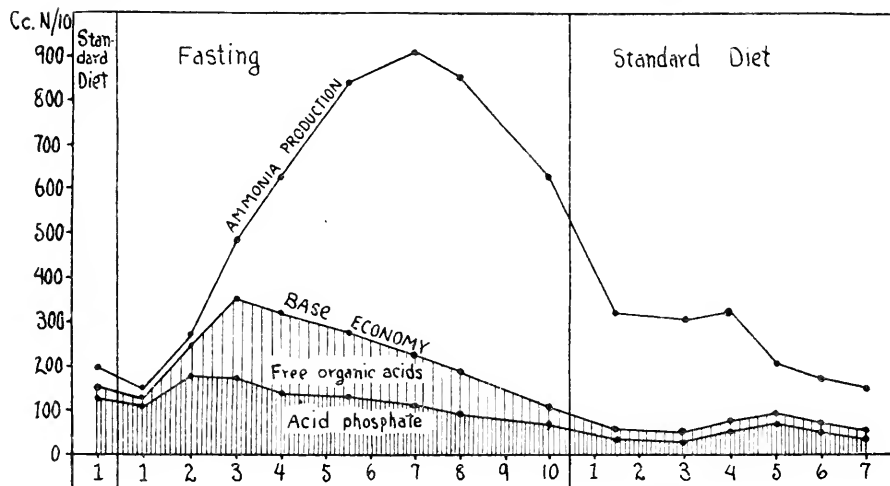


Fig. 6. Presenting graphically the measurements of the factors limiting the excretion of fixed base during a 10 day fast and a fore and after period on a standard diet. Constructed from data given in Tables XI and XIII. The measurements of ammonia production and of base economy are each laid off from the base line. Those of free organic acids and of acid phosphate, which together constitute base economy, are superimposed.

of the phosphates which obtains at pH 6.1 constitutes the ideal position from which to effect a control of base eliminated with phosphoric acid. In illustration of this conception the base economy factor may be described as the "fine" and ammonia production as the "coarse" adjustment of this regulatory mechanism.

To further indicate the relative size of the two factors during the course of a fasting period, the daily values given in Table XI are presented graphically in Fig. 6. The values for base economy and ammonia production are here not superimposed as in Figs. 3, 4, and

5, but are laid off separately from the base line. The value for base economy is, however, divided to indicate the extent to which it is due to the excretion of acid phosphate (in excess of the ratio of acid to alkaline phosphate in the plasma) and of free organic acids, respectively. The data used in plotting this partition of the base economy factor are given in Table XIII.¹³ They are presented to illustrate the fact that during fasting the base saving in the elimination of the organic acids, owing to the huge quantity of them produced, becomes an even larger value than the saving effected in the case of phosphoric acid in contrast with its relatively insignificant size under normal circumstances of metabolism.

VI.

*Data Illustrating the Separate Control of Acid and of
Base Concentrations in the Body Fluids.*

In the diagram (Fig. 1) representing the acid-base composition of the blood plasma, the line between the acid and base values may be taken as representing the practically complete dissociation

¹³ This partition of base economy, *i.e.* the separate estimation of the base saving due to the elimination of phosphoric acid chiefly as BH_2PO_4 and to the excretion of unbound organic acids, was accomplished by calculating the former value on the basis of the total HPO_4'' excretion and urinary pH, and then subtracting it from a measurement of the titratable acidity of the urine, the remainder being taken to represent free organic acids. The base equivalence of HPO_4'' at various degrees of hydrogen ion concentration was computed from Sørensen's data, establishing the $\frac{\text{BH}_2\text{PO}_4}{\text{B}_2\text{HPO}_4}$ ratio which

obtains for a given pH. For example, at pH 7.4, taking (HPO_4'') as 1.0, BH_2PO_4 is 0.2 and (B_2HPO_4) is 0.8. The base (B') bound by phosphoric acid at 7.4 is therefore $0.2 + (0.8 \times 2) = 1.8$ times the molecular concentration of HPO_4'' . Similarly, at pH 5.2 (BH_2PO_4) is 0.98 and (B_2HPO_4) is 0.02 and the base equivalence is $0.98 + (0.02 \times 2) = 1.02$. To illustrate the application of these values: on the 4th day of the fast of F.MeH. the following measurements were obtained from the urine specimen: pH 5.2, HPO_4'' 176 cc. of 0.1 N, and titratable acidity 320 cc. of 0.1 N. From these data the two factors in base economy are calculated as follows:

$$\begin{array}{rcl} 176 \times 1.8 & = & 316 \text{ cc. } 0.1 \text{ N B' bound by } \text{HPO}_4'' \text{ at pH of plasma.} \\ 176 \times 1.02 & = & 180 \text{ " " " " " " " " " " urine.} \end{array}$$

$$\begin{array}{rcl} & & 136 \text{ " } 0.1 \text{ " base economy in elimination of } \text{HPO}_4'' \\ 320 - 180 & = & 140 \text{ " } 0.1 \text{ " " " " " " " " organic} \\ \text{acids.} & & \end{array}$$

of the plasma salts. This line also correctly implies that the acid values may vary independently of the total base concentration. The leveling of the total acid value with that of base by means of an automatic adjustability of the concentration of (HCO_3') has been described in Section II. The processes of acid-base metabolism are thus indicated as proceeding in terms of separately controlled

TABLE XIVa.

Acid-Base Composition of a 48 Hour Urine Specimen From D.M. While Taking 4 Gm. NaCl per Day with a Carbohydrate Diet.

Acids.*		Base.	
	cc. 0.1 N		cc. 0.1 N
Organic acid'	163	NH_4'	659
PO_4'''	77	Mg''	46
SO_4''	142	Ca''	43
Cl'	765	K'	175
		Na'	205
Total	1,147	Total	1,128

* In terms of base bound at pH 5.7, the reaction of the urine specimen.

TABLE XIVb.

Bicarbonate and Chloride in Plasma Before and After Ingestion of NaCl.

	(BHCO_2)	(BCl)
	vol. per cent CO_2	gm. NaCl per 1,000
Before	60	5.77
After	69	5.70

concentrations of ions. The data to be presented here were obtained with the purpose of illustrating this important, though perhaps obvious, proposition by demonstrating that ingested sodium chloride is used not as NaCl but as Na' and Cl' according to the needs of their separate concentrations in the body fluids. These data consist in measurements representing completely the acid-base composition of a 48 hour urine specimen obtained from D. M. during the 4th and 5th days of the carbohydrate period following his 4 day fast; 4 gm. of NaCl per day being ingested during these 2 days. The NaCl was given in butter spread over the toast of the carbohydrate ration and was readily taken. The measure-

ments obtained are given in Table XIV and are also presented graphically in Fig. 7. Their significance is interpreted on the premise that a large loss of body water during the fast, owing to glycogen consumption, was to a considerable extent regained during the period of carbohydrate ingestion. Supporting this assumption

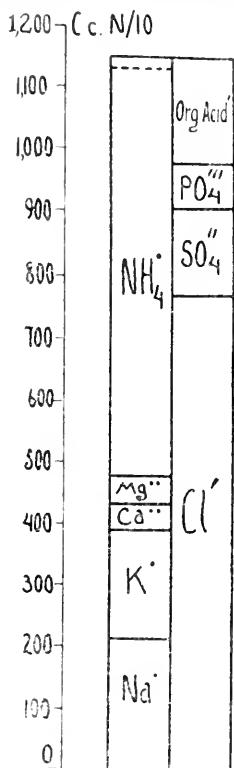


FIG. 7. Presenting graphically the acid-base composition of a 48 hour urine specimen from D. M. while taking 4 gm. of NaCl per day with a carbohydrate diet. The dotted line represents the sum of the measurements of base. Constructed from data given in Table XIV.

body weight measurements showed a loss of 1,840 gm. at the end of the 4 day fast and a gain of 915 gm. during the 5 day carbohydrate period. A demand for retention of the inorganic constituents of the body fluids during the carbohydrate period in order to sustain correct concentrations in an expanding volume of body water is inferred. The most conspicuous acid-base values in the blood plasma are (Na⁺) and (Cl[']). The average usual concentrations per 100 cc. of serum water may be taken as, Na⁺, 158 cc. of 0.1 N and Cl['], 114 cc. of 0.1 N. According to Katz' data the concentrations per 100 cc. of muscle water are, Na⁺, 46 cc. of 0.1 N and Cl['], 26 cc. of 0.1 N. The concentrations of Na⁺ in body water, are, therefore, indicated as larger than those of Cl['], and need for retention of more Na⁺ than Cl['] in the presence of an increase in volume may be postulated. In agreement with these considerations the urine specimen collected during the 2 days that NaCl was given, along with the carbohydrate intake, was found to contain very much more of Cl['] than of Na⁺, the excretion of Na⁺ being 183 cc. of 0.1 N, whereas that of Cl['] was 732 cc. of 0.1 N; approximately half of the 1,380 cc. of 0.1 N Cl['] ingested (see Table XIV). The excretion of Cl['] was

indeed larger than the total fixed base excretion, 402 cc. of 0.1 N, indicating that much of the Cl' given as NaCl entered the urine as NH_4Cl , leaving Na^+ to bind other acids in the body fluids (see Fig. 7). With an increasing volume of body water a prominent value in need of support is bicarbonate. These data from the urine indicate quite certainly that, under the circumstances here present, this ingested salt of a strong acid has contributed base to form the salt of a weak acid, an event which emphatically demonstrates that the secretion in urine of the salts of the plasma is controlled in terms of the individual ions.

Measurements of bicarbonate and of chlorides in the plasma of D. M. directly before and following the 2 days of NaCl administration are also given in Table XIV. It will be seen that (Cl') was approximately sustained and that (BHCO_3) was increased. This increase in bicarbonate is superfluous as evidence of base contributed from NaCl. A stationary value for (BHCO_3) in the presence of an increase in the volume of body water would serve as well to indicate an absolute increase of bicarbonate in the body fluids.

VII.

Acid-Base Values in the Plasma during Fasting.

The data in Section V indicate a careful control of the use of fixed base in the process of acid excretion. The extent to which this regulation of fixed base excretion prevents alteration of normal acid-base values in the body fluids in the presence of the abnormal circumstances of metabolism which obtain during fasting is here considered on the basis of direct measurements of certain values in the plasma.

Measurements of Bicarbonate and of Total Base in the Plasma.—There occurs regularly during fasting a considerable reduction of the plasma bicarbonate which is roughly proportionate to the degree of ketone acid production. At the height of ketosis bicarbonate is found to be reduced by at least one-third of its usual value. In considering the cause of this lowering we have a choice of conjecture based on the outstanding feature of acid-base metabolism during fasting; *viz.*, the huge increase in the excess of acid over fixed base claiming excretion due to the production of the

ketone acids. It may reasonably be surmised that this very abnormally large acid production will overtax the mechanism controlling fixed base excretion with the result that fixed base concentrations in the body fluids will be depleted; at the expense, as has been explained (Section II), of bicarbonate. This is the usually assumed cause of a reduced bicarbonate in the presence of ketosis. The data given in Section V demonstrate, however, a wide extensibility of the factors controlling fixed base excretion. Moreover, the measurements of fixed base in the urine were found to agree fairly closely with the amounts which were roughly calculable as correctly available for use in the process of acid excretion (Section III). A depletion of base concentrations as the cause of bicarbonate lowering should, therefore, not be assumed without the support of direct evidence. The alternate explanation of the bicarbonate reduction is the inference that the rapid production of ketone acids will lead to a considerable accumulation of these acids in the plasma, their base equivalence being supplied at the expense of the normal concentration of bicarbonate. The validity of the first mentioned explanation of the bicarbonate lowering during fasting may be directly tested by measuring the total base of the plasma in the presence of a reduced bicarbonate. In Table XV are given measurements of total base in blood serum obtained from D. M. at the end of his 4 day fast and after 2 days of carbohydrate ingestion. The values for total base consist of the sum of separate measurements of Na, K, Ca, and Mg. The table contains corresponding measurements of bicarbonate in the serum, which show a rather high value at the beginning of the fast, a more than one-third reduction of this value at the end of the 4 day fast, and a return to about the usual value after 2 days of carbohydrate ingestion. The marked degree of ketosis which developed during the fast is indicated by the ketone acid excretion for the 4th day. The values found for total base are seen to be closely stationary and to coincide within the limit of error in obtaining them (taken as ± 4 per cent) with the average usual value; 156 cc. of 0.1 N per 100 cc. of plasma. It is thus evident that the reduction of bicarbonate in this instance is not to a measurable extent referable to depletion of plasma base. It is worth noting, from the point of view of admiring the regulatory mechanism which provides these stationary concentrations, that not only are they maintained in spite of

an at first rapidly mounting and then quickly declining excess of acid over fixed base claiming excretion, but also in the presence of a large shift in the volume of body water, a lowering during the fast, followed by an increase when carbohydrate was given. An appreciation of the alertness of the control of fixed base excretion demanded by these rapidly changing circumstances may be obtained by noting the wide range of the values for fixed base in the urine from this subject plotted in Fig. 5. Since, as these measurements indicate, the lowering of bicarbonate in the presence of ketosis was not in this instance caused to appreciable degree by a reduction of total base, we are led to the inference that it must have been practically entirely a consequence of an accumulation of ketone acids in the plasma. If this be the case, following ingestion of carbohydrate, the concentration of abnormal acids in the plasma should disappear and, total base being intact, bicarbonate should then resume its usual size. The measurement of the ketone acid excretion on the 2nd day of the carbohydrate diet indicates an almost complete disappearance of these acids, and the corresponding measurement of bicarbonate shows it restored to a usual value. The data in Table XV thus constitute satisfactory though indirect evidence that the lowered bicarbonate after 4 days of fasting was due, apparently entirely, to a concentration of ketone acids in the plasma.

The stationary value for total base in the plasma in the presence of a bicarbonate lowering, demonstrated in the case of D. M., is of considerable physiological significance. It may be noted in Table XII and Fig. 5 that the fixed base excretion, although greatly reduced in terms of the total acid excretion, nevertheless, represents a considerable absolute amount. Assuming that 65 per cent of the body weight at the end of the fast may be taken as a measure of the volume of body water, *i.e.* in this case, $18.78 \text{ kilos} \times 0.65 = 12.2 \text{ liters}$; the reduction of base available for binding carbonic acid, which calculated from the data in Table XV was 12.3 cc. of 0.1 N per 100 cc., could have been covered by retention of $12.3 \times 12.2 = 1,500 \text{ cc. of } 0.1 \text{ N base}$. The amount of base which was permitted to enter the urine during the 4 days of fasting was (see Table XII) 3,077 cc. of 0.1 N, double the amount which, if retained, would have provided a normal bicarbonate in the body fluids. It will be at once apparent, however, that compensation

in this manner for base bound in the plasma by ketone acids could not be effected without raising total base above its usual value. It is the evidence of these data that a normal total base is not raised in behalf of a lowered bicarbonate. An accurate maintenance of total base is thus indicated as of more importance physiologically than a stationary concentration of bicarbonate. That this must be the case may be inferred from the close dependence of osmotic pressure values in the body fluids on the size of total base concentrations. It is difficult to imagine compensatory arrangements which could sustain these values correctly in the presence of any considerable alteration of total base. On the other hand, in the case of the relationship of the bicarbonate concentration to the control of reaction in the body fluids, circulatory and respiratory adjustments are capable of compensating correctly over a wide range of bicarbonate lowering. It may be noted from the above considerations that the mechanism regulating fixed base excretion does not operate directly, as is often inferred, in support of the "alkali reserve" (bicarbonate) of the plasma. Were the word permissible in discussing physiological processes, the relationship of the control of fixed base excretion to the size of the bicarbonate concentration might be described as incidental. Given a stationary total base, bicarbonate is determined by the sum of the other acid values in the plasma. As long as these remain closely constant carbonic acid binds a fairly steady allotment of base. Under circumstances, however, causing extension of a normal value¹⁴ or introducing a concentration of abnormal acid the plight of the bicarbonate concentration is apparently entirely ignored by the factors which determine the total fixed base content of the plasma. A further deduction to be noted from the data here given is that glucose rather than bicarbonate is indicated as the logical agent in the treatment of acidosis due to non-diabetic ketosis.

In Table XVI are presented, among other data, measurements of sodium and of bicarbonate in blood serum obtained from A. G.,

¹⁴ In a recently completed study of the effect on acid-base values in the plasma of administering CaCl_2 , NH_4Cl , and HCl to infants with tetany the writers (15,16) found that these substances cause a large extension of the Cl^- concentration in the plasma without appreciably disturbing total base so that there occurs in consequence a closely proportionate reduction of bicarbonate.

before her fast, at the end of 3 day intervals during the 15 day fast, and at the end of the 3 day carbohydrate after period. Since sodium constitutes about 92 per cent of the plasma base, measurements of sodium may be taken as fairly closely indicative of the size of the total base concentration. It will be seen in the table that only at the end of the second 3 day period was the sodium

TABLE XVa.
Fixed Base in Blood Plasma from D.M.

	End of 4 day fast.		End of 2 day carbohydrate after period.	
	mg.	cc. 0.1 N	mg.	cc. 0.1 N
Na'	327.0	142.0	329.0	143.0
K'	22.3	5.7	18.2	4.7
Ca''	10.3	5.2	10.3	5.2
Mg''	2.7	2.3	1.6	1.3
Total		155.2		154.2

Values per 100 cc. of plasma.

For average usual values see foot-note 1, p. 637.

TABLE XVb.
Bicarbonate and Chloride in Plasma.

	(BHCO ₃)	(BCl)
	vol. per cent CO ₂	gm. NaCl per liter
Before fasting	66	6.17
End of 4 day fast	37	5.07
" " 2 " carbohydrate after period	60	5.77

TABLE XVc.
Ketone Acids in Urine.

4th day of fast 5.21 gm. (as β -oxybutyric).
2nd day of after period 0.12 " (" ").

measurement (303 mg. per 100 cc.) definitely below the value (330 mg. per 100 cc.) taken as average normal. The measurements of bicarbonate during the fast, especially at the end of the first two periods, show a very considerable reduction of the initial value and a recovery of even more than this value after 3 days of carbohydrate diet.

Measurements of Ketone Acids in the Plasma.—The concentration of ketone acids was determined in four of the blood samples

TABLE XVI.
Measurements of Sodium, Bicarbonate, Ketone Acids, and Chlorides in Blood Plasma from A.G.

	Before fasting	Fasting					Car- bohy- drate 3rd day.
		3rd day.	6th day.	9th day.	12th day.	14th day.	
Sodium, <i>mg. per 100 cc.</i>	327	324	303	318	324	324	324
Bicarbonate, <i>vol. per cent CO₂</i>	66.4	41.4	45.7	50.4	48.5	52.6	70.0
Ketone acids (β -oxybutyric), <i>gm. per liter</i>		0.82	1.15	0.97		1.21	
(Chlorides, <i>gm. NaCl per liter</i>)	6.03	5.66	5.62	5.47	5.26	5.30	5.90
Bicarbonate, <i>cc. 0.1 N per 100 cc.</i>	29.7	18.5	20.4	22.3	21.7	23.5	31.3
Ketone acids (as β -oxybutyric*), <i>cc. 0.1 N per 100 cc.</i>	(0.0)	7.9	11.0	8.9	(10.3)	11.6	(0.0)
Total	29.7	26.4	31.4	31.2	32.0	35.1	31.3
Chlorides, <i>cc. 0.1 N per 100 cc.</i>	104.0	97.6	97.0	94.7	90.8	91.4	101.8
Total	133.7	124.0	128.4	125.9	122.8	126.5	133.1

* Values in parentheses are assumed.

obtained from A. G. during the course of her fast. These are contained in Table XVI, and together with the bicarbonate measurements are expressed again in the lower part of the table as cc. of 0.1 N per 100 cc. of serum. The relationship of the bicarbonate lowering to the size of the ketone acid concentration is indicated in this table by the fact that the sum of the two concentrations is a roughly constant value of about the magnitude of the values for bicarbonate alone before the fast, and at the end of the carbohydrate after period.

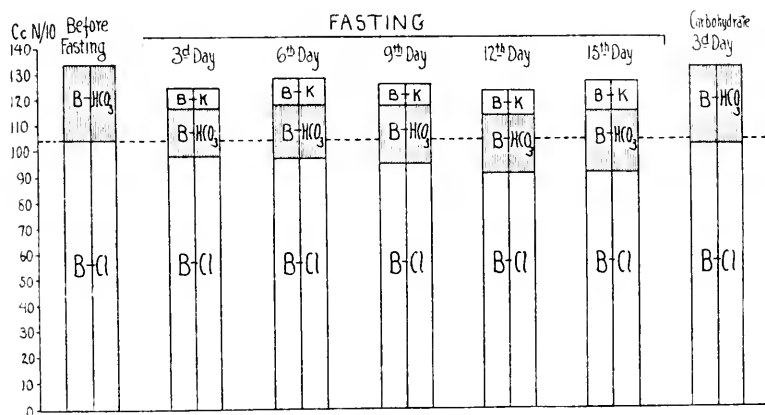


FIG. 8. Presenting graphically the measurements of the concentrations of chloride, bicarbonate, and ketone acids (as β -oxybutyric) given in Table XVI.

Measurements of Chlorides in the Plasma.—The data given above indicate that the reduction of bicarbonate during fasting is largely a consequence of an accumulation of ketone acids in the plasma. The ketone acid concentration does not, however, alone determine the size of the plasma bicarbonate. This would be the case if total base and other acid values in the plasma remained unaltered. The measurements of sodium given in Table XVI indicate, except in two instances, an at least approximately stationary value for total plasma base. They do not, however, dependably demonstrate the absence of a slight lowering. It is conceivable, and indeed probable, that the factors controlling fixed base excretion are only brought fully into play by a fall in plasma base to a certain

critical point. It is the evidence of the data from D. M. (Table XV) that if such a lowering occurs it is a small factor in the reduction of bicarbonate. A much more significant event from the point of view of the bicarbonate concentration is a considerable decline during fasting of the largest acid value in the plasma (Cl'). This is demonstrated by the measurements of plasma chlorides obtained from D. M. and A. G. and given in Tables XV and XVI. Obviously, if total base in the plasma be approximately sustained, reduction of (Cl') will provide a larger bicarbonate than could be the case in the presence of a usual concentration of chloride. From the data in Table XVI, the diagrams in Fig. 8, giving a rough graphic presentation of the relationships of the chief acid values in the plasma during the course of the experiment with A. G., were constructed. The measurements of bicarbonate, ketone acids, and chlorides, given as cc. of 0.1 N per 100 cc. of plasma, are here superimposed. The diagrams will clearly illustrate the extent to which the ketone acid concentration, (B-K), reduces bicarbonate during the fasting period. The size of the contribution to (BHCO_3) due to the decline of (BCl) is shown by the dotted line crossing the diagrams at the level of the top of the value for (BCl) obtained before the fasting period began, and which it may be noted was a closely average value; *viz.*, 6.03 gm. of NaCl per liter. The portion of (BHCO_3) which extends below this dotted line in the diagrams may be taken as existing by grace of recession of (BCl), and it may be seen that during the latter part of the fasting period nearly half of the base bound by (HCO_3') was at the outset of the fast covered by (Cl'). These diagrams illustrate again the mendicant position of (HCO_3') among the other acid values demanding base in the plasma.

The circumstances which cause this decrease in (BCl'), which so opportunely augments (BHCO_3'), are not apparent. May it be regarded as a controlled adjustment in favor of (BHCO_3) or is it simply a consequence of an unavoidable depletion of Cl' concentrations in the body water? As regards the latter possibility it may be pointed out again that Cl' being presumably present within the body, only in solution in the body water, an extremely small amount of Cl' becomes available during fasting for replacement of Cl' entering the urine, the only source of Cl' being from the water of destroyed protoplasm, where, according to Katz' muscle tissue

measurement, it is held at a concentration of only 26 cc. of 0.1 N per 100 cc. as compared with 114 cc. of 0.1 N per 100 cc. in the water of blood serum. Phosphoric acid and sulfuric acid, owing to their production by the oxidation of protein, are much more abundantly supplied. That this chief acid of the plasma is available during fasting to a very limited extent is indicated by the fact that during the course of a fasting period the urine becomes almost chlorine-free (see Tables X and XI and Figs. 3 and 4). During the first several days of fasting the chloride excretion is relatively very large, presumably because of a rapid reduction of the volume of extracellular water (see Fig. 2). It is conceivable that, with the subsequent decline of ketosis, the level of glycogen content in the tissues may rise and tend to cause an increase in the volume of both intra- and extracellular water in proportion to the protoplasmic mass. Under such circumstances the Cl' becoming available from destroyed protoplasm might not be adequate for maintenance of (BCl') in the plasma. As regards this surmise it should be noted that the availability of Na' during fasting is almost as slender as that of Cl', and yet its concentration in the plasma is apparently successfully sustained (see Tables XV and XVI). According to Katz' measurements there is, however, to a slight degree relatively less of Cl' than of Na' in muscle water, as compared with serum water; the Cl':Na' ratio being 0.57 as compared with 0.72 in the case of serum water. Although they do not provide basis for a clear inference on this point the following Cl':Na' ratios obtained from the urines collected from these children during the early periods of their fasts may be presented; A. G., first 6 days of fast, 0.63; D. M., 4 days of fasting, 0.65; M. C., first 4 days of fast, 0.54. It will be seen that the first two of these ratios lie between those given for Cl':Na' in the plasma and in muscle water and suggest a correct control of Cl' elimination during the period of rapid reduction of the volume of body water. The chlorides of the plasma, nevertheless, fell during these periods, in the case of A. G. from 6.03 to 5.62 gm. as NaCl per liter, and of D. M., from 6.17 to 5.07 gm. The ratios in the urine for the remainder of the fasts of A. G., and of M. C. are 1.60 and 0.84, respectively, suggesting a wastage of Cl'. The absolute amounts of Na' and Cl' entering the urine during these periods are, however, so small that an inference drawn from their ratio is scarcely dependable.

On the whole these data would seem to constitute at least negative evidence against the surmise that the decline of (BCl) in the plasma is due to a failure of control of Cl' elimination.

It is a permissible conjecture that the lowering of (BCl) in the plasma may be due to an alteration of the distribution of Cl' in the body water. This possibility is indicated by the mobility of Cl' in the blood (Zuntz's phenomenon) in contrast with the permanently extracellular position of its chief companion ion Na'. The fact that a rise in the (BCl) of the plasma was observed to occur following carbohydrate ingestion very definitely suggests the probability that the reduced (BCl) during fasting is due to an altered distribution rather than to an absolute depletion of the Cl' content of the body water. In the case of A. G. the plasma chlorides at the end of the 15 day fast were 5.30 gm. per liter (as NaCl). After 3 days of carbohydrate diet they were found to be 5.90 gm. Corresponding values obtained from D. M. were 5.07 gm. after 3 days of fasting, and 5.77 gm. after 2 days of carbohydrate ingestion. Obviously, this rise of (BCl) in the plasma is not concordant with an explanation of the previous lowering on the basis of an absolute depletion of (Cl'). Moreover, it was found that during the carbohydrate after periods much more Cl' than Na' was excreted in the urine, the Cl':Na' ratios for these periods being 2.8 and 3.3 in the case of A. G. and of D. M., respectively; the ratio increasing when NaCl was added to the diet of D. M. to 4.0. These data suggest that contrary to the impression obtained from the measurements of (Na') and (Cl') in the plasma the body's store of Cl' is better conserved during fasting than is its content of Na'.

VIII.

SUMMARY.

The total fixed base concentration may be regarded from a purely structural point of view as the most important value in the body fluids. A correct ionic concentration is closely dependent on the maintenance of a usual concentration of fixed base, and hydrogen ion concentration is also, but more flexibly, in relationship with this value. In this study preservation of an approximately stationary concentration of fixed base is taken as the chief adjust-

ment of the processes of acid-base metabolism. The premise is used that the concentrations of fixed base in various compartments of body water are roughly of the same size as may be measured in blood plasma. A few data supporting this surmise are cited in Section II.

Regarding fixed base (except in the case of calcium) as entirely in solution at a uniform concentration in body water, the extent to which it may be used correctly in the process of acid excretion during fasting will be determined by the degree of reduction of the volume of body water. Body water is lost during fasting in consequence of two events, the destruction of protoplasm with release of its water content and a reduction in tissue glycogen causing a decrease in cell volume. Supporting this very simple conception of the source of available fixed base during fasting, calculated values for fixed base claiming excretion in consequence of the reduction of the volume of body water are found to agree satisfactorily with the amounts of fixed base measured in the urine when allowance is made for calcium in the urine estimated as from calcium deposits. These data (Section III) explain the source of fixed base used in the process of acid excretion during fasting without the necessity of hypothecating the existence in the body of "base depots" except in the case of calcium. They cannot, however, be taken as of a precision to permit the inference that there is no appreciable variation in the distribution of total fixed base in the body water. Attempt is also made in Section III to evaluate the factors of body water excretion on the basis of measurements of sodium and potassium in the urine. The rough data obtained serve to illustrate the possibility of using such measurements as a means of studying changes in the volume of body water.

The excretion of inorganic acids during fasting is composed of the acid radicles contained in the lost body water and in addition those derived by oxidation from protein. The inorganic acid excretion is also closely proportional to the excretion of organic acids by reason of an approximately stationary ratio of protoplasm destroyed to body fat consumed (Section IV).

The factors which control the use of fixed base in the process of acid excretion and thereby sustain base concentrations in the body fluids are described, and their operation during fasting is illustrated in quantitative terms in Section V.

That the concentration of fixed base in the body fluids may be regarded as a structural entity is indicated by the data given in Section VI, demonstrating a separate control of the excretion of Na⁺ and of Cl⁻ in the urine following ingestion of NaCl.

Measurements of base in the plasma during fasting (Section VII) demonstrate that the factors controlling fixed base excretion sustain an approximately correct concentration. The reduction of the plasma bicarbonate is to be practically entirely referred to an accumulation of ketone acids. It is to be noted that total base in the plasma is not raised above its usual value in behalf of a lowered bicarbonate. A very considerable decrease in the concentration Cl⁻ in the plasma was observed to occur during the course of periods of fasting. A large part of the concentration of bicarbonate found in the plasma during fasting is due to this recession of (Cl⁻).

Chemical Methods Used.

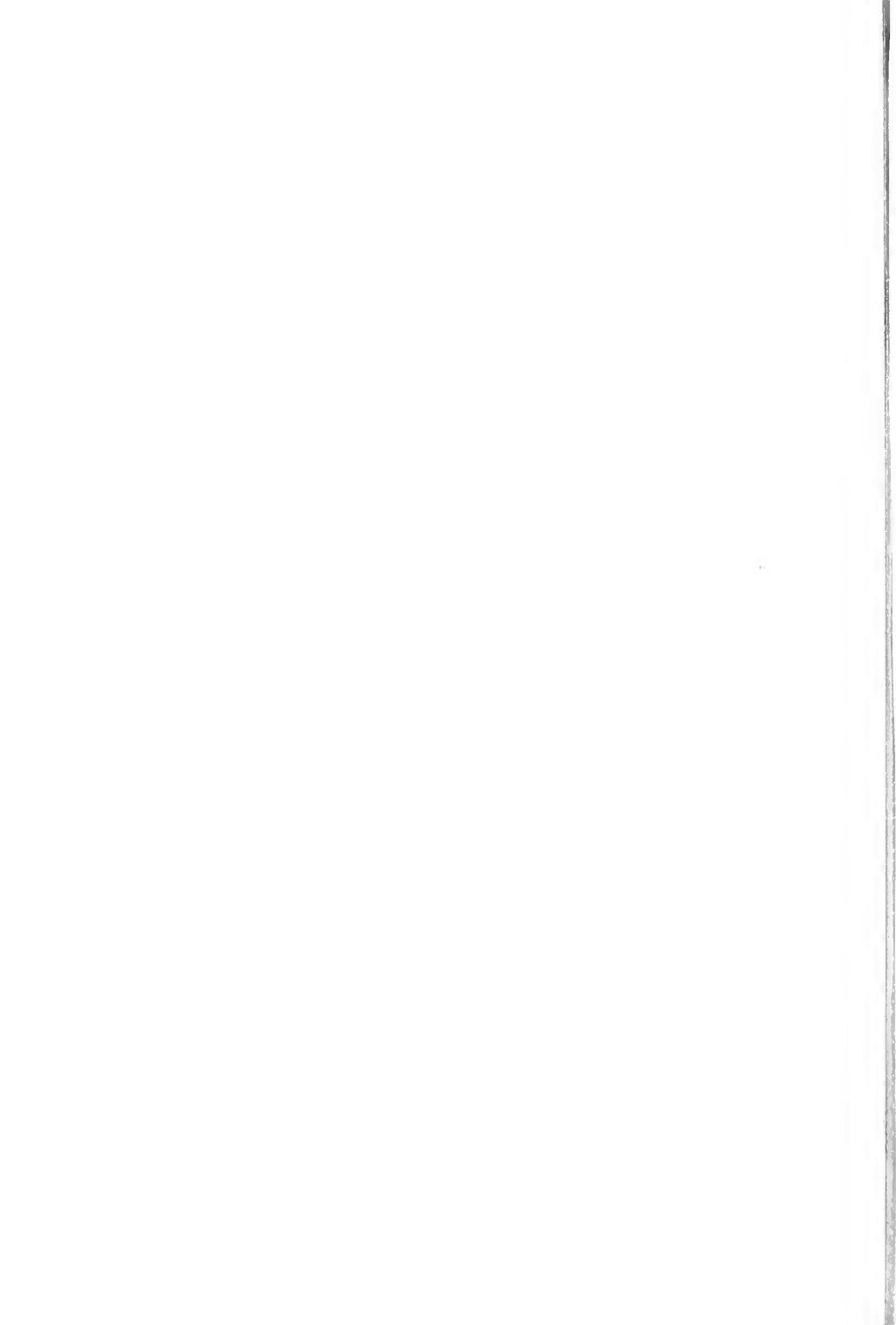
Sodium, potassium, calcium, and magnesium were determined in blood serum and in urine by the methods of Kramer and Tisdall (17). For chlorides in blood serum a combination of Van Slyke's plan of precipitating the protein with picric acid and Volhard's thiocyanate titration was used. This unpublished procedure was devised by D. W. Atchley and is essentially the same as that recently described by Myers and Short (18). Bicarbonate in the serum was determined by Van Slyke's method (19), and Van Slyke's procedure (20) for measuring the ketone acids in the serum was also used. Serum protein was estimated on the basis of refractometric readings. The pH and titratable acidity of the urine were determined by the methods of Palmer and Henderson (21). The total organic acid excretion in the urine was measured by the titration procedure devised by Van Slyke and Palmer (22). The values found were corrected for creatinine and creatine on the basis of measurements of these substances by the method of Folin (23). Ketone acids were measured either by the method of Shaffer and Marriott (24) or that of Van Slyke (25), the urine samples being first thoroughly aerated with the purpose of removing acetone. Urine phosphates (inorganic) were determined by the uranium acetate titration method and urine sulfates (inorganic) were weighed as barium sulfate according to the method of Folin. Ammonia was determined in the urine by the method of Folin and MacCallum (26) and urine nitrogen by the usual Kjeldahl method.

The urine was collected in all of the experiments as 24 hour specimens, and the measurements of pH, titratable acidity, total organic acids, ammonia, creatinine, and creatine were obtained during the day following the collection period. Chloroform was used as a preservative and the bottle receiving the urine was stoppered and shaken after the addition of each voiding.

The blood plasma values given in this paper were, in most instances, measurements obtained from samples of blood serum. Only those of bicarbonate and of chlorides in Tables XIV and XV are from the plasma of oxalated blood. "Plasma," being for the purposes of discussion the more suitable term, is used throughout, and the very slight error contained in the assumption that plasma values may be measured in serum samples is neglected. The blood samples were in all instances collected in a Luer syringe and then delivered, through small bore glass tubing, at the bottom of a 15 cc. centrifuge tube. The tube was rapidly *filled to the top* and immediately stoppered. Loss of CO₂ is, we have found, quite as efficaciously prevented by this method of collection as by delivering the blood under a layer of oil.

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STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

VI. THE INFLUENCE OF THE PROTEIN OF THE DIET ON THE SYNTHESIS AND RATE OF ELIMINATION OF HIPPURIC ACID AFTER THE ADMINISTRATION OF BENZOATES.

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In a previous paper (1) it was shown that the rate of synthesis of hippuric acid in rabbits after benzoate feeding was markedly increased by the simultaneous administration of glycine with the benzoate but was not affected by the administration of alanine, leucine, nor-leucine, isovaline, cystine, or aspartic acid. It was concluded that the increased output of hippuric acid after the ingestion of benzoate and glycine was due to the presence in the organism of large quantities of preformed glycine. Since there was no increase after the feeding of these other amino-acids, the latter were not considered to be readily available precursors of glycine. If these amino-acids were typical in this respect, then the feeding of proteins with the sodium benzoate should increase the hippuric acid excretion in proportion to the glycine content of the proteins. Experiments in support of this conclusion are reported in this paper.

That the hippuric acid output after benzoate administration is affected by the protein intake has been both affirmed and denied. Parker and Lusk (2) observed no change in the hippuric acid excretion of rabbits after feeding benzoate alone and benzoate with 4 to 10 gm. of casein or gelatin. However, these investigators used the 24 hour output of hippuric acid as a measure of hippuric acid synthesis, a procedure which would not detect changes in the rate of synthesis. The latter has been shown to

vary decidedly with the amount of available glycine present (1). Ringer (3) found that fasting rabbits formed hippuric acid as readily as normal rabbits but Magnus-Levy (4) came to the opposite conclusion. Raiziss and Dubin (5) reported that, following the ingestion of benzoate, rabbits excreted more hippuric acid on a milk and egg diet than on a vegetable diet. They believed that a high protein level favored the synthesis of hippuric acid.

The ingestion of proteins high in glycine content with benzoate might readily increase the rate of synthesis of hippuric acid because of their preformed glycine content. However, if proteins low in glycine increased the rate of synthesis of hippuric acid, it would appear that precursors of glycine were produced by the reactions occurring in the general metabolism of proteins. Certain individual amino-acids, such as alanine or leucine, evidently are not easily converted into glycine (1). The ingestion of proteins of varied amino-acid content would give an opportunity to determine the importance for glycine synthesis of other amino-acids than those previously studied and in addition the effect of increasing the level of general protein metabolism.

EXPERIMENTAL.

The procedure used in these experiments was the same as that employed in the earlier experiments with individual amino-acids (1). Rabbits were fed benzoate alone and benzoate with protein and the elimination of hippuric acid in the following 6 hour period was determined. It has already been demonstrated that the rate of excretion of hippuric acid following the administration of moderate amounts of benzoate is a measure of the rate of the synthesis of hippuric acid and that this rate is directly influenced by the supply of glycine available for synthesis.

In view of the fact that the rate of digestion of different proteins might introduce a variable factor, the proteins were hydrolyzed *in vitro* with pepsin and then with trypsin for 2 to 3 weeks. This had the added advantage of converting the insoluble proteins into soluble hydrolytic products which were easily fed. Digests of gelatin, casein, edestin, dried egg albumen, elastin, glutenin, and peanut meal were prepared. The gelatin, casein,

and egg albumen were commercial products. The edestin, glutenin, and peanut meal were prepared in this laboratory. The elastin preparation was made by grinding a fresh ligamentum nuchæ from the ox and subjecting this material to enzymatic hydrolysis. Its protein content was calculated from the data of Vandegrift and Gies (6). In addition to these proteins a commercial peptone preparation was also used. The glycine content

TABLE I.
*Glycine Content of the Various Proteins Fed.**

Protein.	Glycine.
	<i>per cent</i>
Elastin.....	25.8
Gelatin.....	25.5†
Edestin.....	3.8
Glutenin.....	0.9
Peptone (Witte's).....	0.8
Casein.....	0
Egg albumin.....	0
Arachin (peanut).....	0‡

* Except where noted the figures are quoted from the compilation given by Plimmer, R. H. A., *Chemical constitution of the proteins. Part I*, London, 3rd edition, 1917, 111 to 135.

† Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

‡ Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 491.

of these substances is shown in Table I. Inasmuch as the methods for the quantitative estimation of glycine are inadequate, these figures must be considered as minimal and comparative only.

Free benzoic acid was determined by the method of Raiziss and Dubin (7) and total benzoic acid by the method of Kingsbury and Swanson (8). Hippuric acid or combined benzoic acid was calculated by subtracting the free benzoic from the total benzoic acid. The rabbit conjugates moderate doses of benzoic acid almost entirely as benzoyl glycine, benzoyl glycuronate being present in traces only, if at all. In Tables II and III, corrections have been made for the daily normal excretion of hippuric acid, so that the figures represent extra free and combined benzoic acid. The quantity of hydrolyzed protein administered with the benzoate was equivalent to 5.0 gm. of the

original protein. This quantity has approximately the same nitrogen content as 3.5 to 4 gm. of glycine, which amount was three molecular equivalents of the benzoate fed.

DISCUSSION.

The results of the experiments on eight of the nine rabbits tabulated in Table II show strikingly that gelatin and elastin, the two proteins characterized by a high glycine content, were the only proteins which noticeably increased the rate of synthesis of hippuric acid when administered with benzoate. Attention should be called to the fact that this same effect was obtained with gelatin as well as with hydrolyzed gelatin. No noticeable difference between the two in their effect on the rate of synthesis of hippuric acid could be observed. For example, in the 6 hour period after the ingestion of benzoate alone, Rabbit 46 (Table II) excreted 41 per cent as hippuric acid; after benzoate and glycine, 76 per cent; and after benzoate and gelatin, 71 per cent. On the other hand, after the ingestion of casein and peptone with benzoate, only 40 and 45 per cent, respectively, were excreted as hippuric acid.

The results with Rabbit 40 (Table II) are particularly interesting because it was possible to complete the whole series of experiments on this animal. After the feeding of gelatin, elastin, and glycine with benzoate, this rabbit excreted 64, 75, and 74 per cent, respectively, as hippuric acid. After the administration of benzoate with casein, edestin, glutenin, peanut meal, egg albumen, and peptone, all glycine-poor proteins, the hippuric acid output was practically the same as after the ingestion of benzoate alone, 47 to 51 per cent. That the increased synthesis of hippuric acid was due to the glycine content of the elastin and gelatin was demonstrated by the experiment in which the quantity of glycine present in 5.0 gm. of gelatin (about 1 mol) was substituted in place of an equivalent amount of nitrogen in a protein containing no glycine. This gave a mixture approximately equal to gelatin or elastin in its content of nitrogen and of glycine. Thus, 5.0 gm. of egg albumen with benzoate had no effect, while 4.0 gm. of the same protein plus only $\frac{3}{4}$ mol of glycine produced a marked increase in the hippuric acid output.

TABLE II.

Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate Alone, of Sodium Benzoate with Glycine, and of Sodium Benzoate with Proteins, Both Native and Hydrolyzed.

Date	Benzoic acid.					Notes.
	Weight.	Excreted.		Ingested.		
		Free.	Combined.			
Rabbit 45.						
1923	kg.	gm.	gm.	per cent of intake	gm.	
Jan. 17	1.9	0.094	0.605	32	1.9	Control.
" 20	1.9	0.005	1.290	68	1.9	5.0 gm. gelatin.
" 23	1.9	0.045	1.003	53	1.9	5.0 " nutrose.
" 26	1.9	0.078	1.202	63	1.9	5.0 " casein (hydrolyzed).
" 31*	1.7	0.110	0.821	43	1.9	5.0 " " (").
Rabbit 46.						
Jan. 20	2.2	0.005	1.513	76	2.0	Glycine (2 equivalents).
" 23	2.2	0.006	1.419	71	2.0	5.0 gm. gelatin.
" 25	2.2	0.078	0.815	41	2.0	Control.
" 27	2.2	0.053	0.793	40	2.0	5.0 gm. casein (hydrolyzed).
Feb. 3	2.2	0.012	0.908	45	2.0	5.0 " peptone.
" 6	2.2	0.002	1.511	76	2.0	5.0 " gelatin (hydrolyzed).
Rabbit 47.						
Jan. 20	2.2	0.078	0.803	40	2.0	Glycine (2 equivalents).
" 23	2.2	0.001	1.484	74	2.0	5.0 gm. gelatin.
" 25	2.2	0.074	0.903	45	2.0	Control.
" 27	2.2	0.074	0.778	39	2.0	5.0 gm. casein (hydrolyzed).
Feb. 2	2.2	0.022	0.843	42	2.0	5.0 " " "
" 14	2.2	0.126	1.050	53	2.0	5.0 " gelatin (hydrolyzed).
" 16	2.2	0.276	0.565	28	2.0	4.4 " casein (hydrolyzed). Benzoate administered 3½ hrs. after casein.
" 21	2.2	0.482	1.089	54	2.0	5.0 gm. gelatin (hydrolyzed). Benzoate injected intravenously 1 hr. after gelatin.
" 24	2.2	0.318	1.258	63	2.0	Glycine (3 equivalents). Benzoate injected intravenously.
Mar. 10	2.0	0.067	0.794	40	2.0	Control.

* Rabbit was fasted for 3 days preceding this experiment.

TABLE II—*Continued.*

Date.	Weight.	Benzoic acid.			Notes.
		Excreted.		Ingested.	
		Free.	Combined.		
Rabbit 40.					
1923	kg.	gm.	gm.	per cent of intake	gm.
Mar. 24	2.0	0.028	0.850	47	1.8 Control.
Apr. 11	2.1	0.002	1.128	64	1.8 5.0 gm. gelatin.
" 13		0.002	1.020	57	1.8 5.0 " casein (hydrolyzed).
May 12	2.1	0.050	0.897	50	1.8 5.0 " edestin (hydrolyzed).
June 6	2.1	0.090	0.741	41	1.8 5.0 " glutenin (hydrolyzed).
" 8	2.2	0.063	1.358	75	1.8 5.0 " elastin (hydrolyzed).
" 11	2.2	0.210	0.805	45	1.8 5.0 " peanut meal (hydrolyzed).
" 15	2.3	0.037	0.883	49	1.8 5.0 " egg albumen (hydrolyzed).
" 17	2.3	0.000	1.325	74	1.8 Glycine (3 equivalents).
" 19	2.3	0.011	1.226	68	1.8 4.0 gm. egg albumen (hydrolyzed) and glycine (0.75 equivalent).
" 22	2.2	0.051	0.943	52	1.8 5.0 gm. casein (hydrolyzed).
" 24	2.2	0.005	0.924	51	1.8 Control.
" 26	2.2	0.011	1.017	56	1.8 5.0 gm. peptone.
" 28	2.2	0.000	1.270	71	1.8 5.0 " elastin (hydrolyzed) and glycine (2 equivalents).
July 1†	2.2	0.000	0.888	49	1.8 100 cc. milk-egg-sugar mixture.
" 3‡	2.1	0.000	0.842	47	1.8 Fasting.
" 4	2.0	0.000	0.721	40	1.8 "
" 6	1.9	0.000	0.655	36	1.8 "
" 14§	2.1	0.000	0.845	47	1.8 Milk-egg-sugar mixture.
Rabbit 54.					
June 21	1.5	0.000	0.855	47	1.8 Control.
" 23	1.5	0.018	1.207	67	1.8 4.0 gm. elastin (hydrolyzed).

† For 2 days preceding this experiment the rabbit was fed daily a mixture containing 200 cc. of milk, 20 gm. of sucrose, and 1 egg.

‡ Rabbit was fasted from July 2 to 7.

§ On the day previous to this, the rabbit received 200 cc. of milk, 20 gm. of glucose, and 1 egg. On the experimental day the rabbit was fed a mixture of 100 cc. of milk, 20 gm. of glucose, and 2 eggs in two equal portions; the first, 3 hours before the benzoate administration, and the second with the benzoate.

TABLE II—*Concluded*

Date.	Weight.	Benzoic acid.		Ingested.	Notes.
		Excreted.			
		Free.	Combined.		

Rabbit 54—Concluded.						
1923	kg.	gm.	gm.	per cent of intake	gm.	
June 25	1.5	0.090	0.981	54	1.8	4.0 gm. edestin (hydrolyzed).
“ 27	1.5	0.136	0.932	52	1.8	4.0 “ casein (hydrolyzed).
“ 30	1.5	0.005	1.131	63	1.8	2.0 “ “ (“) and glycine (0.9 equivalent).
July 2	1.6	0.024	1.385	77	1.8	Glycine (4 equivalents).
“ 5	1.6	0.057	0.755	42	1.8	5.0 gm. egg albumen (hydrolyzed).

Rabbit 55.						
June 20	2.5	0.000	0.888	49	1.8	Control.
“ 22	2.5	0.011	1.105	62	1.8	Glycine (4 equivalents).
“ 23	2.4	0.057	1.251	70	1.8	5.0 gm. elastin (hydrolyzed).

Rabbit 56.						
June 20	2.4	0.011	0.678	34	2.0	Control.
“ 22	2.4	0.024	1.200	60	2.0	Glycine (3 equivalents).
“ 23	2.4	0.162	1.127	56	2.0	5.0 gm. elastin (hydrolyzed).
“ 25	2.5	0.063	0.873	44	2.0	5.0 “ glutenin (hydrolyzed).
“ 28	2.5	0.142	0.798	40	2.0	5.0 “ casein (hydrolyzed).
“ 30		0.000	1.095	55	2.0	4.0 “ glutenin (hydrolyzed) and glycine (0.8 equivalent).
July 3	2.6	0.057	0.881	44	2.0	5.0 gm. egg albumen (hydrolyzed).

Rabbit 57.						
June 25	2.7	0.000	0.700	35	2.0	Control.
“ 30	2.6	0.052	1.100	55	2.0	4.0 gm. peanut meal (hydrolyzed) and glycine (0.8 equivalent).
July 2	2.6	0.184	0.761	38	2.0	5.0 gm. peanut meal (hydrolyzed).

Rabbit 62.						
July 9	2.0	0.000	0.937	52	1.8	Glycine (3 equivalents).
“ 10	1.9	0.040	0.620	34	1.8	Control.
“ 11	1.8	0.017	0.764	42	1.8	5.0 gm. edestin (hydrolyzed).
“ 18		0.046	0.911	51	1.8	4.0 “ “ (“) and glycine (0.8 equivalent).

In our earlier experiments (1) it was found that usually 3 mols of glycine produced the maximum increase in the hippuric acid output, smaller amounts very often being without effect, and larger quantities being without any additional effect. Both of these points are illustrated in the present series of experiments. In the case of Rabbit 47 (Table II), 2 mols of glycine were insufficient whereas 3 mols produced the characteristic increase. On the other hand, while both glycine and elastin markedly increased the hippuric acid output of Rabbit 40 (Table II), no greater increase was observed when the glycine and elastin were fed together. In these experiments with proteins, less than 1 mol of glycine was enough to increase the hippuric acid output provided a large excess of other amino-acids was present in the organism. These other amino-acids seemed to aid by sparing the preformed glycine ingested and not necessarily by giving rise to precursors of new glycine. In no case was the increase in the glycine eliminated in conjugation with benzoic acid greater than the quantity of glycine fed. Even with the ingestion of only $\frac{3}{4}$ mol of glycine as in the egg albumen experiment cited above, more glycine was administered than was excreted as benzoyl glycine in the 6 hour period.

It is important to note that at widely separated intervals and after many administrations of benzoate there was no appreciable change in the quantity of hippuric acid excreted after the ingestion of benzoate alone. On March 24, Rabbit 40 detoxicated 47 per cent of the ingested benzoate in the 6 hour period and on June 24, 51 per cent. Similarly, Rabbit 47 detoxicated 39 per cent on January 27, and 40 per cent on March 10.

The results of the experiments with the other animals of the series were similar to those already discussed in detail with the exception of Rabbit 45 (Table II). This animal showed an increased hippuric acid output in one experiment with hydrolyzed casein and a less marked increase in one experiment with undigested nutrose (commercial sodium caseinate). However, after 3 days of fasting, casein feeding did not cause an increased hippuric acid output. At the time of the administration of benzoate alone, this rabbit was in poor condition and the benzoate produced very severe toxic effects. Unfortunately, the

rabbit was not available for a second control feeding of benzoate. The relatively small synthesis of hippuric acid after the first benzoate administration may have been due to the toxic symptoms produced. The results of the ingestion of casein and benzoate in eight experiments on five other rabbits failed to show any marked differences from those of the ingestion of benzoate alone.

Raiziss and Dubin (5) reported that rabbits fed 200 cc. of milk, 20 gm. of sucrose, and 1 egg per day showed an increased hippuric acid output after benzoate administration. Rabbit 40 (Table II) was given this diet for 2 days and on the 3rd day was given benzoate with half of the above diet. The benzoate excreted as hippuric acid in this experiment was the same as that after benzoate feeding on normal days. Later this experiment was repeated with a still higher protein intake. On the experimental day the rabbit received one-half of a mixture of 100 cc. of milk, 20 gm. of glucose, and 2 eggs, 3 hours before the benzoate administration and the remainder at the same time as the benzoate administration. As before, there was no increase above the percentage after benzoate on normal days. This experiment allowed a time interval of almost 9 hours for the ingested protein, which was low in glycine, to give rise to precursors of glycine. A similar experiment was performed with Rabbit 47. Benzoate was fed $3\frac{1}{2}$ hours after 4.4 gm. of casein. In this case also the longer period failed to result in an increased hippuric acid synthesis.

Rabbit 40 (Table II) was fasted for 5 days and during this period the percentage of ingested benzoate excreted as hippuric acid in 6 hours decreased from 47 to 36 per cent. This result could not be confirmed with another rabbit. The study of the effect of fasting on the rate of synthesis of hippuric acid is being continued.

In order to afford further evidence concerning the influence of proteins and the level of protein metabolism on the rate of synthesis of hippuric acid, the effect of the administration of thyroid was studied. The ingestion of thyroid is generally considered to result in an increased protein metabolism and it was considered that the increased protein catabolism occasioned by the thyroid might result in a stimulation of hippuric acid synthesis, either

directly by the liberation of greater amounts of glycine from the tissues, or indirectly by mass action of protein catabolites. The thyroid (Armour's desiccated) was fed in daily doses of 0.6 and 1.0 gm. The ingestion of thyroid (Table III) by two rabbits

TABLE III.

Effect of Thyroid Feeding on the Excretion of Free Benzoic Acid and of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Benzoate.

Date.	Weight.	Benzoic acid.			Ingested.	Thyroid powder fed.
		Excreted.				
		Free.	Combined.			
Rabbit 54. Diet: 250 cc. of milk per day.						
1928	kg.	gm.	gm.	per cent of intake	gm.	gm.
June 21	1.50	0.000	0.855	47	1.8	None.
July 7	1.65				None.	"
" 8	1.65	0.023	0.779	43	1.8	0.6
" 9	1.65				None.	0.6
" 10	1.60				"	0.6
" 11	1.45	0.017	1.051	58	1.8	0.6
" 12	1.40				None.	0.6
" 13	1.35	0.017	1.243	69	1.8	0.6
" 16	1.30	0.058	0.860	48	1.8	None.
Rabbit 40. Diet: 250 cc. of milk per day.						
Mar. 24	2.00	0.028	0.850	47	1.8	None.
June 24	2.20	0.005	0.924	51	1.8	"
July 15	2.15				None.	1.0
" 16	2.05	0.000	0.150	64	1.8	1.0
" 17	2.00				None.	1.0
" 18	1.90	0.000	0.159	64	1.8	1.0
" 19	1.90				None.	None.
" 20	1.80				"	"
" 21	1.80	0.029	0.938	52	1.8	"

resulted in an increase in the 6 hour output of hippuric acid after the ingestion of benzoate. In both animals, administration of the benzoate 3 days after the cessation of the thyroid feeding showed a return to the original (control) rate of synthesis. Further experi-

ments on the effect of thyroid are in progress and will be reported later as will the nitrogen eliminations of the present series.

The experiments reported in this paper have been in confirmation of our earlier work (1). They indicate that, under the conditions of these experiments at least, the main factor which limits the rate of synthesis and elimination of hippuric acid is the amount of preformed glycine available in the organism. If the metabolism of protein in general results in the formation of glycine precursors or of glycine itself, this effect was not manifested under the conditions of our experiments. The ingestion of proteins which had a low content in preformed glycine almost uniformly failed to influence the synthesis of hippuric acid.

SUMMARY.

1. The rate of synthesis of hippuric acid in rabbits was markedly increased after the oral administration of sodium benzoate with hydrolyzed elastin and gelatin, proteins which have a high content of preformed glycine.

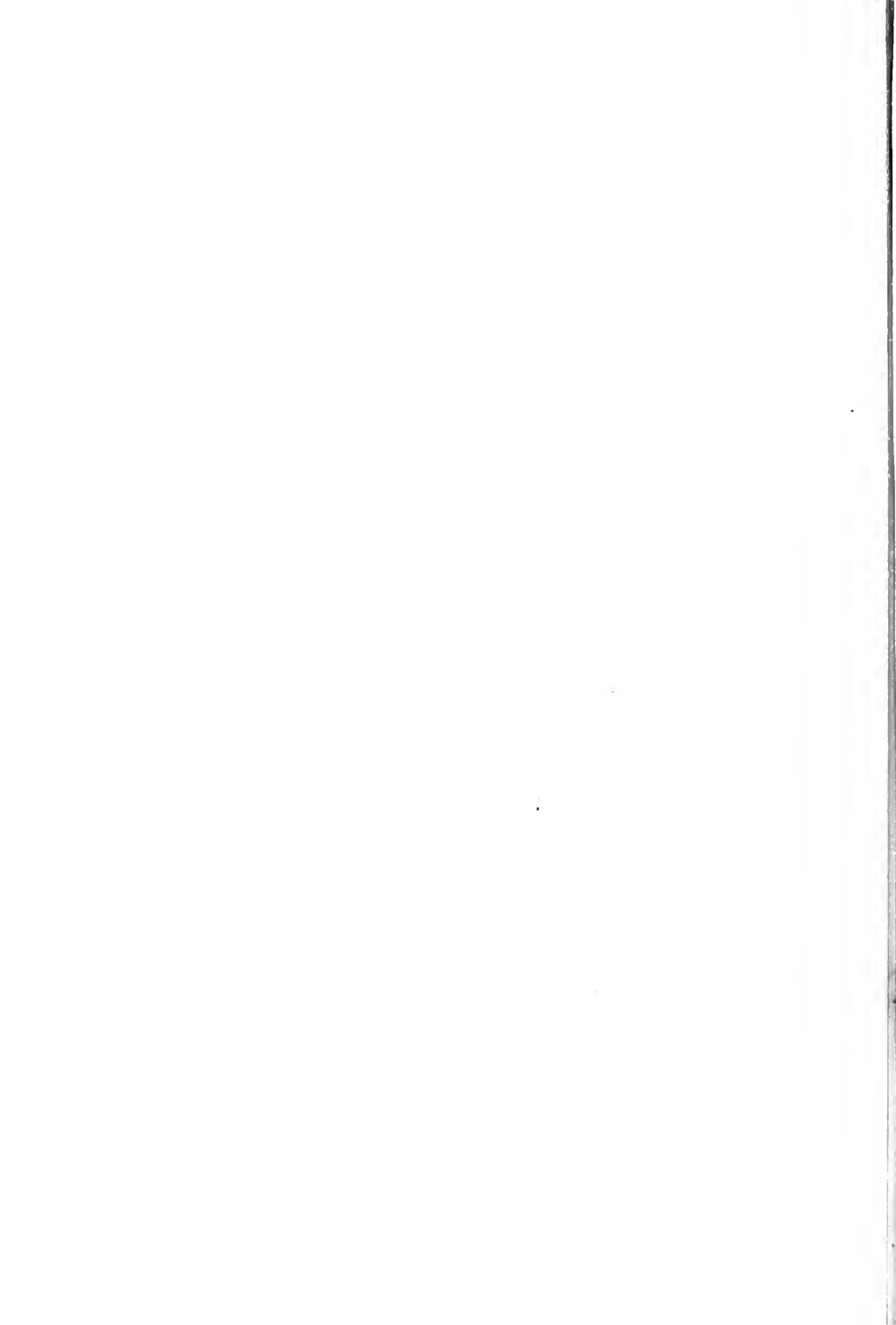
2. The administration of peptone and of hydrolyzed edestin and glutenin, which have a low content of glycine, and of hydrolyzed casein, egg albumen, and peanut meal, which are almost entirely lacking in glycine, did not increase the rate of synthesis of hippuric acid after benzoate feeding.

3. The administration of small amounts of glycine with hydrolyzed casein, edestin, egg albumen, glutenin, and peanut meal did cause an increase in the rate of synthesis of hippuric acid after the ingestion of benzoate.

4. It is considered improbable that the ordinary reactions of general protein metabolism produced readily available precursors of glycine.

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THE PREPARATION OF INSULIN.

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When the Insulin Committee suggested that we should report on the recent progress in the preparation of insulin an extensive review of the history of pancreatic extracts was at first contemplated. Macleod (1), Dale (2), and others, however, have recently reviewed certain parts of the literature, and since a study of that portion of this literature which describes the preparation of the extracts shows that many of them are of minor significance in the present connection, we have decided to refer only to those investigators whose work, in our opinion, led them very near to the solution of the problem.

Zuelzer (3), a German investigator, was one of the early workers in this field. He prepared extracts from pancreas in several ways, one of which is as follows: The pancreas was minced and sufficient sodium bicarbonate was added to produce a weak alkaline reaction. The mixture was then left to autolyze for several days. (This procedure in our experience results in a great loss of potency of the material. Zuelzer stated, however, that this step might be omitted.) The liquid was then pressed out and alcohol added until no more albuminous substances were precipitated. When the solution was free from albumin, it was filtered and concentrated in a vacuum still. The final product obtained, Zuelzer says, was a fine, dry, gray powder. This powder, he states, was easily soluble in water or dilute alkali; was free from ferments; and gave none of the known protein reactions. Insulin, as we know it at present, is less stable in alkaline than in acid solution. The purest preparation we have as yet obtained from mammalian pancreas gives a positive biuret reaction.

Zuelzer tested his preparation by determining to what extent it antidoted the hyperglycemia and glucosuria caused by administration of epinephrine. Von Fürth and Schwarz (4) and others have reported that many other substances counteract the effect of epinephrine.

Zuelzer's extract was administered to several diabetic patients, and in certain of these cases was partially successful, in his own hands, in alleviating the symptoms of diabetes. In the hands of others (Forschbach, 5), however, the toxic effects overshadowed the beneficial action to such an extent that further treatment was abandoned.

Although Zuelzer nearly discovered in 1908 the active pancreatic principle which we call insulin, his work in the light of subsequent events must be considered an abandoned research.

E. L. Scott (6), working on the hypothesis that later was independently formulated by Banting, narrowly missed demonstrating in 1912 the internal secretion of the pancreas. He was unable to secure complete atrophy of the acinous cells of the pancreas in dogs, after attempting to ligate the pancreatic ducts. He did not report the effect of administration of extract of this partially atrophied gland. Scott also tried to extract the internal secretion by means of alcohol, but in his endeavor to inhibit the action of the external secretion, he raised the concentration of alcohol to such a height that a large proportion of the active principle was undoubtedly never extracted from the gland. The alcoholic extract obtained from the gland was concentrated *in vacuo* and the residue extracted with ether. The ether extract was discarded. The residue was dissolved in 95 per cent alcohol. Since insulin from mammalian pancreas is only slightly soluble in alcohol of this concentration, Scott could have had very little of the active substance in his final extract. In the preparation of watery extracts Scott used a preliminary alcoholic extraction. The concentration of alcohol was approximately 85 per cent for the first extraction of the glands. At this concentration a part of the insulin would go into solution. This was evidently discarded. A large part of the active substance would be left behind, however, and should have been present in the second extract which was obtained by treating the glands with water. Possibly one reason why better effects were not obtained with this material was that an inhibiting substance which is at least partially precipitated by 80 per cent alcohol, must have been present in large amounts in the watery extracts.

Rennie and Fraser (7) in 1907 studied the effects on diabetic patients of the oral administration of the principal islets of certain bony fishes. In one case these investigators administered a saline extract of islet tissue by subcutaneous injection. No beneficial influence on the symptoms of diabetes was observed. Since insulin in a purified form has not as yet been administered successfully by mouth, we have not far to look for the cause of failure of those experiments in which the crude islet tissue was orally administered. The subcutaneous injection of the extract of the islet tissue produced very profound symptoms of toxicity.

Knowlton and Starling (8) hazarded the opinion that the pancreatic hormone "would be a body diffusible, soluble in water, unstable in alkaline solution, but more stable in slightly acid solution, and not destroyed immediately at the temperature of boiling water." It is interesting to note that these speculations (with the exception of that one relating to the diffusibility of the substance) are correct. We have not as yet obtained a preparation which dialyzes through parchment. The diffusibility of the active principle is a property which has to be retested every time a purer preparation is obtained. We have carried out the procedure used by these investigators in the preparation of their extract, and have demon-

strated the presence of insulin in the resulting mixture. The tests are much more conclusive, however, if the crude extract so obtained is purified in any one of the several ways which we will attempt to outline in the body of this paper. The work of Knowlton and Starling (8) and of Paterson and Starling (9) has been reviewed in several communications by the Toronto group (10). We merely wish to point out here that had these investigators used criteria other than the change in sugar consumption of the perfused mammalian heart to determine the potency of their material, more encouraging results might have been obtained.

Murlin and Kramer (11) in 1913, prepared alkaline extracts of pancreas. The administration of this extract reduced the degree of glycosuria in diabetic dogs. It was discovered, however, that this effect was produced by the administration of alkali alone. With the exception of experiments in which the respiratory quotients of several diabetic animals were studied, no further work was reported by Murlin and his collaborators, until after the publication of the initial experiments of the Toronto investigators.

Kleiner (12) showed that the administration to diabetic dogs of unfiltered watery extracts of fresh pancreas which had been diluted with 0.90 per cent sodium chloride, usually resulted in a marked decrease in blood sugar. A decrease in the amount of sugar excreted in the urine accompanied the reduction of the blood sugar level. Kleiner suggested that the diminished excretion of sugar might be partly due to the toxic renal effects. Kleiner's experiment was repeated and confirmed by Banting and Best (10). Anuria was observed, however, in the experimental animal.

Very significant results have been obtained by a group of workers who have attempted to demonstrate in blood or in pancreatic perfusates a substance necessary for the proper combustion of carbohydrates in the body. The most important of these contributions are those of A. H. Clark (13), Lépine (14), Drennan (15), Hédon (16), Forschbach (17), and Murlin and Kramer (11). Since we contemplate reporting on the demonstration of insulin in blood, a review of the work of these investigators will perhaps be more advantageously included in that communication.

Preparation of Earlier Extracts.

In July, 1921 (10), extracts were prepared in the Department of Physiology of the University of Toronto, which were shown to contain the internal secretion of the pancreas. In the preparation of this extract the degenerated pancreas was removed from dogs 10 weeks after ligation of the pancreatic ducts. The gland was obtained as soon as possible after the death of the animal and was sliced into a chilled mortar containing Ringer's solution. The mortar was placed in a freezing mixture and the contents were partially frozen. Sand was added and the tissue was completely pulverized. The temperature of the contents of the mortar was then raised and the resulting liquid filtered through paper. The filtrate was administered intravenously to diabetic dogs. The results of the injection of this material have been published (10). Extracts were prepared by

this simple procedure from the normal pancreas of the dog and from fetal calf pancreas. The normal pancreas did not yield as much of the active principle per gram of tissue as did the degenerated gland. The pancreas of the fetal calf, however, provided comparatively large quantities of the substance. It would be interesting to compare the amounts of active material obtainable from these sources, if the tissues were treated with a high acid extractive, as in our present procedure. This causes the immediate inactivation of proteolytic enzymes and a comparatively efficient extraction of the active principle.

Banting and Best (18) prepared active extracts from the normal pancreas of the ox by extraction of the gland with alcohol, which had been made acid by the addition of 0.2 per cent hydrochloric acid. The concentration of alcohol in the mixture was in some cases as high as 60 per cent. The liquid was removed from the glands by filtration and the filtrate evaporated either in porcelain dishes placed in a warm air current or by means of a laboratory vacuum still. The liquid was usually evaporated so that from 5 to 10 cc. contained one dose for a depancreatized dog. This material was administered to several diabetic dogs. One animal, in particular, Dog 33, which lived 70 days after pancreatectomy, received many injections of this preparation. Rigid aseptic precautions were not observed in the administration of the extract to this animal. However, so long as daily doses of active material were administered there was little or no suppuration at the sites of injection. Numerous abscesses developed when insulin was omitted. These disappeared, however, when the treatment was resumed. The improvement in the clinical condition of the animals receiving this material was very marked. The respiratory quotients of completely diabetic dogs were definitely raised and large amounts of glycogen¹ were deposited in the livers of the animals when glucose and insulin, which had been prepared by the above procedure, were administered. In some cases the filtrate referred to was evaporated to dryness and the residue extracted with toluene to remove the lipoid material. This procedure caused no loss in potency. *These investigators were able to demonstrate that the active principle contained in this residue was practically insoluble in 95 per cent ethyl alcohol.* They treated an aliquot portion of the dried residue with 95 per cent alcohol. The mixture was filtered and the filtrate evaporated. The residue was dissolved in saline solution. Administration of this solution produced no effect upon the blood or urinary sugar of a depancreatized dog. A saline solution of the material which did not dissolve in 95 per cent alcohol, however, definitely lowered the blood sugar and diminished the sugar excretion of the same animal. A watery solution of this material was passed through a Berkefeld filter, but a considerable loss of potency was observed. Trieresol in excess of that amount used as a preservative in biological products, such as diphtheria antitoxin, did not injure the active substance. Material prepared by these investigators from beef pancreas was administered to several diabetic

¹ *Tr. Roy. Soc. Canada*, 1922, xvi, 6.

patients in the clinic of the Toronto General Hospital. A decrease in blood sugar and a lowered sugar excretion resulted. A certain degree of local irritation was observed in some of the cases. This was probably due to the high percentage of protein present in the extract. The results of these experiments showed that insulin could be derived from a readily available source—beef pancreas; that a preparation could be secured from this source which was efficient in completely removing the symptoms of diabetes from depancreatized animals; and that these results, as far as the effects on hyperglycemia and glucosuria were concerned, could be duplicated in the clinic. The practical application of the results was very evident.

A method for the further purification of insulin was evolved principally by J. B. Collip who joined forces with the discoverers of insulin in December, 1921. This method has been reported (19). The details are briefly as follows: 95 per cent alcohol was added to freshly minced pancreas so that the volumes of extractive and glands were approximately equal. After an interval of a few hours, during which the mixture was stirred, the liquid was filtered off. 95 per cent alcohol was added to the filtrate to secure a concentration of approximately 80 per cent alcohol. The mixture was then filtered and the filtrate concentrated *in vacuo*. The vacuum was secured by the use of a laboratory water pump. When the liquid had been concentrated to a small volume the lipid substances were removed by extraction with ether. The watery solution was then further concentrated to a pasty consistency. This material was treated with 80 per cent alcohol and the mixture centrifuged. The active principle was found to be contained entirely in the alcohol which formed the uppermost layer in the centrifuge tube. The alcoholic solution was removed and was added to several volumes of 95 per cent or absolute alcohol. The active principle, as mentioned above, was practically insoluble in alcohol of this concentration. The precipitate obtained by the above procedure was removed by a Buchner filtration, dissolved in distilled water, and concentrated to remove traces of alcohol and to secure the desired concentration of active substance. The preliminary clinical effects of this preparation have been reported (20). This method of purification worked out satisfactorily, for a short time, on a small scale. Larger scale experiments were not successful and subsequently it was found impossible to duplicate consistently the earlier results on any scale. For a period of 2 months scarcely any insulin was available. A method was then evolved, however, in this laboratory, with the assistance of various members of the Toronto group, which utilized the facts reported by Banting and Best and many of the details of Collip's procedure. The method gave consistent results, and furnished all the insulin used in Toronto for clinical and experimental work for a period of 3 months prior to the beginning of collaboration with Eli Lilly and Company. This method was as follows: Minced pancreas was extracted with an equal volume of 95 per cent acetone. A small amount of formic or acetic acid was added. Collip had previously found formic acid advantageous. The concentration never exceeded 0.1 per cent. (Higher acid was first used in this laboratory as a result of

a conversation with H. H. Dale, in which the acidity of the extractive was discussed.) The pancreas acetone mixture was allowed to stand for several hours and was then filtered. The filtrate was placed in enamel lined trays (500 cc. to each tray measuring $18 \times 18 \times 2.5$ inches). The trays were placed in a tunnel through which a current of hot air was rapidly drawn. The 500 cc. were evaporated to approximately 50 cc. in about 1 hour. The temperature of the liquid never exceeded 35°C . The residue was removed from the trays, chilled to 0°C ., and filtered. Lipoid material was largely eliminated in this manner. The filtrate thus obtained was treated with 95 per cent ethyl alcohol to secure a concentration of approximately 80 per cent alcohol. The mixture was then filtered and the filtrate added to 5 or more volumes of 95 per cent alcohol as in the previous method. The precipitate which formed was allowed to settle to the bottom of the alcoholic solution. From 24 to 48 hours were allowed for the precipitate to settle. The alcohol was then decanted off and the precipitate dissolved in distilled water. Traces of alcohol were removed by vacuum distillation. This was the method originally adopted and applied to larger scale production by Eli Lilly and Company when information regarding the production of insulin was communicated to them by the Toronto Committee.

One of the first contributions made by the scientific staff of Eli Lilly and Company was the employment of rotary high vacuum pumps which immediately made possible the efficient concentration of the original acetone or alcoholic filtrate and also of the filtrate after the 80 per cent alcoholic precipitation.

Benzoic Acid Method.

This method of preparing and purifying insulin was evolved in these laboratories by Moloney and Findlay (21). The principle of this process is based on the fact that certain substances readily adsorb insulin. The particular adsorbing substance used by these investigators was benzoic acid. The method briefly is as follows:

Minced pancreatic glands were extracted with alcohol and the filtrate was concentrated in an efficient vacuum still. To each liter of the crude aqueous concentrate 50 cc. of a 25 per cent sodium benzoate and 12.5 cc. of concentrated hydrochloric acid were used. These amounts were usually sufficient to saturate the solution with benzoic acid. However, these quantities can be increased or decreased proportionately depending on the amount necessary to cause a first lasting precipitate. Then to this saturated benzoic acid solution 40 cc. of 25 per cent sodium benzoate and 10 cc. of concentrated hydrochloric acid were added. The precipitate thus formed was allowed to settle and the solution filtered. This precipitate usually contained about two-thirds of the potent material. The filtrate was again treated with 40 cc. of sodium benzoate and 10 cc. of hydrochloric acid to secure a second precipitate. This precipitate was filtered off and the filtrate again treated if this was considered necessary. The benzoic acid precipitates were mixed and added to a small volume of

80 per cent ethyl alcohol which dissolved both the insulin and benzoic acid. Certain inert materials, however, settled out and the alcoholic solution was filtered. The filtrate was concentrated to dryness *in vacuo* and the benzoic acid dissolved by treatment with ether. This solution was transferred to a separatory funnel and a small volume of water was added. The insulin was contained in the aqueous layer.

The introduction of the benzoic acid method of purification marked a distinct advance in the production of insulin. By this process the large amounts of alcohol necessary for the fractional precipitation of the proteins and the final precipitation of the insulin in the previous methods were avoided. Chemically, it gave a product which was much freer from protein material, as determined by the nitrogen content, than anything we had hitherto been able to obtain. Clinically, the toxic and indurating effects which characterized all the earlier extracts were greatly reduced. The main disadvantage which this process possessed was that the separation of the benzoic acid precipitates often required long and tedious filtration. However, the benzoic acid method has been a very important factor in the production of insulin over a considerable period of time. This is shown by the fact that approximately 250,000 units of insulin made by that method under our direction in this laboratory were used clinically in Toronto in the autumn of 1922, with very satisfactory results.

Water Extracts.

In the past many attempts have been made to obtain the substance necessary for the utilization of carbohydrates in the body by watery extraction of the pancreas. Knowlton and Starling, as previously stated, prepared an extract from the pancreas by extracting the gland with acidulated cold water. Shortly after the original publication by the Toronto group, Sansum² was able to obtain a small quantity of insulin by hot water extraction of beef pancreas. Because of the possible economic significance of a watery extraction of the pancreas in the manufacture of insulin, this method has been investigated in our laboratories. Some 150 experiments using different modifications in the extraction, such as varying the time, the temperature, the acidity, etc., have been carried out. While all these experiments are of interest, we will report only a few which have given the most promising results.

2 pounds of minced beef pancreas were added to 300 cc. of distilled water which had been acidified with 4 cc. of concen-

² Sansum, W. D., Unpublished work.

trated sulfuric acid. After 20 minutes 1 liter of boiling water was added to the mixture, and the temperature raised to 80°C. by a jet of live steam. This temperature was maintained for a period of 2 minutes. The mixture was then poured into a flask and cooled quickly by connecting the flask to a high vacuum pump. The cooled contents were filtered. An almost colorless filtrate was obtained. After the completion of the filtration which usually took about $\frac{1}{2}$ hour, the glands were reextracted with 1 liter of acidified water at room temperature for a period of 3 hours. The liquid was filtered off as in the first extraction. The insulin in the combined filtrates was purified either by the method elaborated by Banting, Best, Collip, and Macleod, or by the present method of purification used in our laboratories.

We were able to obtain equally satisfactory results by cold water extraction of the pancreatic glands. The method was as follows: 2 pounds of minced pancreas were added to 1,500 cc. of distilled water which was acidified with 3.5 cc. of concentrated sulfuric acid. The mixture, after extraction for 2 hours, was filtered through fluted filter paper. The filtrate was quite clear and had a pH of approximately 3.5. It is very essential that this pH be very close in the above value for two reasons. The acidity is outside the isoelectric range of many of the proteins in the pancreas, and at this hydrogen ion concentration there is obtained a mixture which filters readily and gives a clear filtrate. The glands were reextracted with acidified water, as above, for 2 hours and the liquid filtered off as in the first extraction. The insulin in the combined filtrates may be purified by any of the methods described in this paper.

Under conditions as described above we were able to obtain fairly satisfactory yields of insulin (see p. 720). The results, however, though very encouraging, have not as yet shown nearly as great a unitage per pound of pancreas as that obtained by the alcohol or acetone method of extraction under the best experimental conditions.

The Method of Doisy, Somogyi, and Shaffer (22).

These investigators have described a method of purification of insulin, the salient new features of which were the precipitation of insulin from watery solution with half saturation of ammonium sulfate and the so

called isoelectric precipitation. Ammonium sulfate in one-half saturation had been previously used in these laboratories by Moloney. Full details of the isoelectric precipitation of insulin from watery solutions containing the active substance were communicated to us by Prof. P. A. Shaffer, and almost immediately afterwards, by Dr. Clowes of Eli Lilly and Company. This method was evidently worked out independently in two laboratories at about the same time. We have profited by discussion of this method with Prof. Shaffer and Dr. Clowes on several occasions. The experimental work in the research laboratories of Eli Lilly and Company was carried out by G. Walden under the direction of Dr. Clowes. The crude material, to which the "isoelectric" method of purification was applied, was obtained by different procedures by the two groups of investigators. It appears from experiments we have carried out that insulin can be removed from watery solution at various hydrogen ion concentrations by procedures which cause a precipitate to settle out. For example, the addition of copper sulfate to obtain a concentration of 1 per cent, in a solution of insulin at pH 3.7 causes the separation of a precipitate which may contain much of the potent material. Similarly, if insulin is added to a solution of edestin and the hydrogen ion concentration adjusted to 6.89, the isoelectric point of this protein, a precipitate forms which may contain all the potency of the original solution.

The Present Method.

In our present process we have employed various steps of many of these methods. The precipitation of insulin from alcoholic solution by the addition of ether was suggested to us by an experiment performed by H. W. Dudley, in this laboratory. In this experiment Dudley demonstrated that the addition of an equal volume of ether to the alcohol used in the final precipitation of insulin in the procedure of Banting, Best, Collip, and Macleod, resulted in a much more efficient precipitation of the active principle than that obtained by the use of alcohol alone.

Fresh pancreatic glands from the ox are obtained from the abattoirs. After separating as much of the fat and connective tissue as possible the glands are placed in large containers which are collected every 3 hours and taken to the laboratory.

The glands are weighed. They are then run through a power meat chopper in which they are finely minced. This minced material is poured into large earthenware crocks which contain a weight of 95 per cent denatured alcohol (10 per cent methyl and the remainder ethyl), equal to that of the glands. The alcohol is acidified to 1.3 per cent with acetic acid. It is important that

a high hydrogen ion concentration be secured at this stage. It inhibits the action of proteolytic enzymes and affects the proteins in such a way as to facilitate separation of the solid and liquid materials at a later stage of the process. Sulfuric acid may be used in place of acetic acid, but, if so, a more highly colored filtrate is obtained. This color is difficult to remove at a later stage. The minced glands are extracted for 3 hours in this acid alcohol solution. During this time they are slowly agitated in order to facilitate extraction. At the end of 3 hours this alcoholic mixture is poured into a rotary centrifuge to separate the alcoholic extract from solid materials. After the completion of the centrifuging the solid material remaining in the centrifuge is re-extracted for 3 hours with a volume of 60 per cent alcohol equal to that of the liquid removed after the first extraction. The alcoholic extract, after 3 hours, is separated by means of the centrifuge. The extracts from the first and second extractions are mixed, neutralized to litmus with sodium hydroxide, and chilled in a brine tank to 0°C. (the chilling may be omitted). During the chilling the filtrate becomes turbid due to the separation of lipid and protein materials. The mixture is filtered through large glass funnels which have been fitted with fluted filter papers. The alcoholic extract thus obtained is almost colorless. The filtrate which contains the active principle is concentrated to about one-twentieth of its original volume in an efficient vacuum still. During the distillation the temperature is not allowed to rise above 30°C. The reasons for this are as follows: excessive heat will coagulate much of the protein material. This is undesirable at this stage because some of the insulin would be adsorbed on the precipitated proteins. Excessive heat over the period required for the concentration produces highly colored decomposition products which greatly increase the difficulty of purification of insulin. After the completion of the distillation the concentrate is quickly heated to 55°C. At this temperature lipid and other materials rise to the surface and are readily skimmed off. This fatty mass which contains about one-quarter of the total potency of the concentrate is treated with sufficient ether to dissolve the lipid material and is allowed to stand over night. The ether is then removed and the residue made up to 80 per cent with denatured alcohol. This mixture is filtered through paper.

Ammonium sulfate is added to the liquid portion of the concentrate to secure half saturation (37 gm. per 100 cc.). This mixture is stirred well and almost immediately protein material separates out and readily rises to the top of the liquid. After standing $\frac{1}{2}$ hour the protein precipitate is skimmed off and allowed to drain on hardened filter paper for 3 to 6 hours. It is then added to sufficient 95 per cent alcohol to secure a final concentration of 75 to 80 per cent alcohol. The amount of alcohol added is usually very small, but varies with the amount of moisture held in the protein precipitate. Much of the protein material is precipitated by this concentration of alcohol and is removed by filtration. This filtrate is mixed with that obtained when the residue from the fatty mass (which was extracted with ether), is treated with 80 per cent alcohol, as described above. The active principle in these combined filtrates is precipitated by adding to them an equal volume of sulfuric ether. On standing over night this precipitate settles to the bottom of the flask and the ether-alcohol solution is decanted. The precipitate is brought to dryness *in vacuo* and is then treated with dilute ammonium hydroxide of such a concentration that the pH of the resulting solution is approximately 8. At this pH the insulin is completely soluble. The hydrogen ion concentration is then adjusted to a pH of 3.5. At this hydrogen ion concentration a precipitate containing dark colored material usually separates out. This is removed by filtration. The filtrate which is an aqueous extract containing the active principle may be pure enough for clinical use. However, it is advisable to purify it further either by the so called isoelectric precipitation (22), by Dudley's picrate method, or by the use of charcoal. This latter method of purification has been worked out by J. P. Moloney and D. M. Findlay in this laboratory and has been found very satisfactory. This method will be published by these investigators at an early date. The purified product is diluted with acidified water (pH 2.5) to the desired potency as estimated by the rabbit test.

After determining the strength of the insulin, 0.1 per cent tricresol is added, and the solution is passed through a Mandler filter. The insulin, after passing through the filter and before the vials are filled, is retested carefully to determine its potency. It is then diluted with sterile distilled water, pH 2.5, so that it

contains 10 or 20 units per cubic centimeter. The method of standardizing insulin has been described elsewhere (23). The tested insulin is poured into sterile glass vials with aseptic precautions, and the sterility of the final product thoroughly tested by approved methods.

Yields of Insulin.

The unit of insulin has recently been defined in several communications. It is one-third the amount of material required to lower the blood sugar of a 2 kilo rabbit, which has been fasted 24 hours, from the normal level (0.118 per cent) to 0.045 per cent over a period of 5 hours.

The earlier extracts obtained from the degenerated pancreas of the dog, normal dog's pancreas, or the pancreatic tissue of fetal calves, were tested upon diabetic dogs. It is difficult, therefore, since the relative susceptibility of depancreatized dogs and normal rabbits to insulin has not been accurately determined, to quote definite figures in rabbit units for the yield of insulin originally obtained per gram of these tissues.

Pork pancreas has consistently given us somewhat larger yields in experimental lots than has beef pancreas. Beef glands are, however, somewhat easier to process, because they have adherent a smaller amount of fat. They have been used exclusively in this laboratory for the production of larger quantities of material.

During the early part of April, 1922, the yield of insulin suitable for clinical use was approximately 15 units per kilo of pancreas. Later in the same month we were able to obtain about 40 units of purified material per kilo. Experimental lots at that time showed as high as 90 units of crude insulin per kilo. Our present procedure, as previously described, gives a yield of approximately 400 units of purified material per kilo. *The increase in acidity of the extractive has been the greatest single factor in improving the yields.*

The yields secured by watery extraction of the glands are extremely promising. The extraction with boiling water under the most favorable conditions gives a consistent yield of approximately 225 units per kilo. The results of extraction by cold, highly acidified water are even more interesting, at the present

time, than those from hot water. Extracts obtained by these procedures are at present more difficult to purify than those obtained by alcoholic extraction.

The highest yields we have as yet obtained were secured from beef pancreas by alcoholic extraction. In several experiments (15 pounds of pancreas were used in each experiment) we have been able to obtain approximately 900 units of purified insulin per kilo of pancreas. In the preliminary experiments of this series, however, large volumes of extractive were used, and we are not certain as yet that the procedure will be practical. The material was purified in some cases by the benzoic acid method, and in others by the ammonium sulfate and isoelectric method. Recent results tend to show that the volume of the extractive may be greatly diminished without lowering the yield if certain precautions are observed.³

DISCUSSION.

During the year 1922, those of us who were responsible for the preparation of insulin for clinical use had insufficient opportunities for the systematic investigation of the chemical properties of the material we were struggling to prepare for patients who were being treated by our clinical collaborators. Changes in the method of production were rapidly introduced, and in many cases were discarded after a brief trial. As our knowledge of the properties of the material has increased, improvements in the method have been introduced.

Alcohol was the extractive used by the original investigators in the University of Toronto in the preparation of insulin from beef pancreas. At many times in the past and especially very recently it has appeared that water would be a more economical solvent. To increase the number of units of insulin obtainable per kilo of pancreas or to introduce a cheaper extractive and thus to assist in lowering the cost of insulin is very desirable, but the question of yields must always be subsidiary to that of the purity of the product. In consideration of this latter point, we believe at the present time that alcohol is the most preferable extractive we have yet investigated.

³ These experiments have been carried out with the assistance of W. J. Grant and will be reported shortly.

The active interest of the members of the Department of Physiology, Biochemistry, and Pharmacology, in our work has been a very important factor in our progress. We have benefited by the suggestions of the representatives of the British Medical Research Council who visited our laboratory. An ingenious method of purification evolved by Dudley⁴ which has been extensively used in England, promises to be of use in the preparation of a dry powder. Insulin in this form seems to be very stable. The collaboration of the investigators mentioned in the body of this communication has been greatly appreciated. The research staff of Eli Lilly and Company has played a prominent part in the rapid development of efficient methods for large scale production.

It has been our intention to review the methods used in Toronto for the preparation of insulin, and not to discuss in detail the properties of this substance. However, certain obvious characteristics of the material are discernible from a study of the various procedures used in the preparation. The stability of insulin is of particular interest and suggests that further research may result in a more highly purified product being obtained.

We regret that we have not had the opportunity to test thoroughly various procedures for the preparation and purification of insulin which have been developed by Prof. T. Brailsford Robertson and Prof. A. B. Anderson of the University of Adelaide, Australia, and by Prof. August Krogh of Copenhagen University, Denmark. The details of these procedures were communicated to Prof. J. J. R. Macleod, and we are indebted to him for very promptly making them available to us.

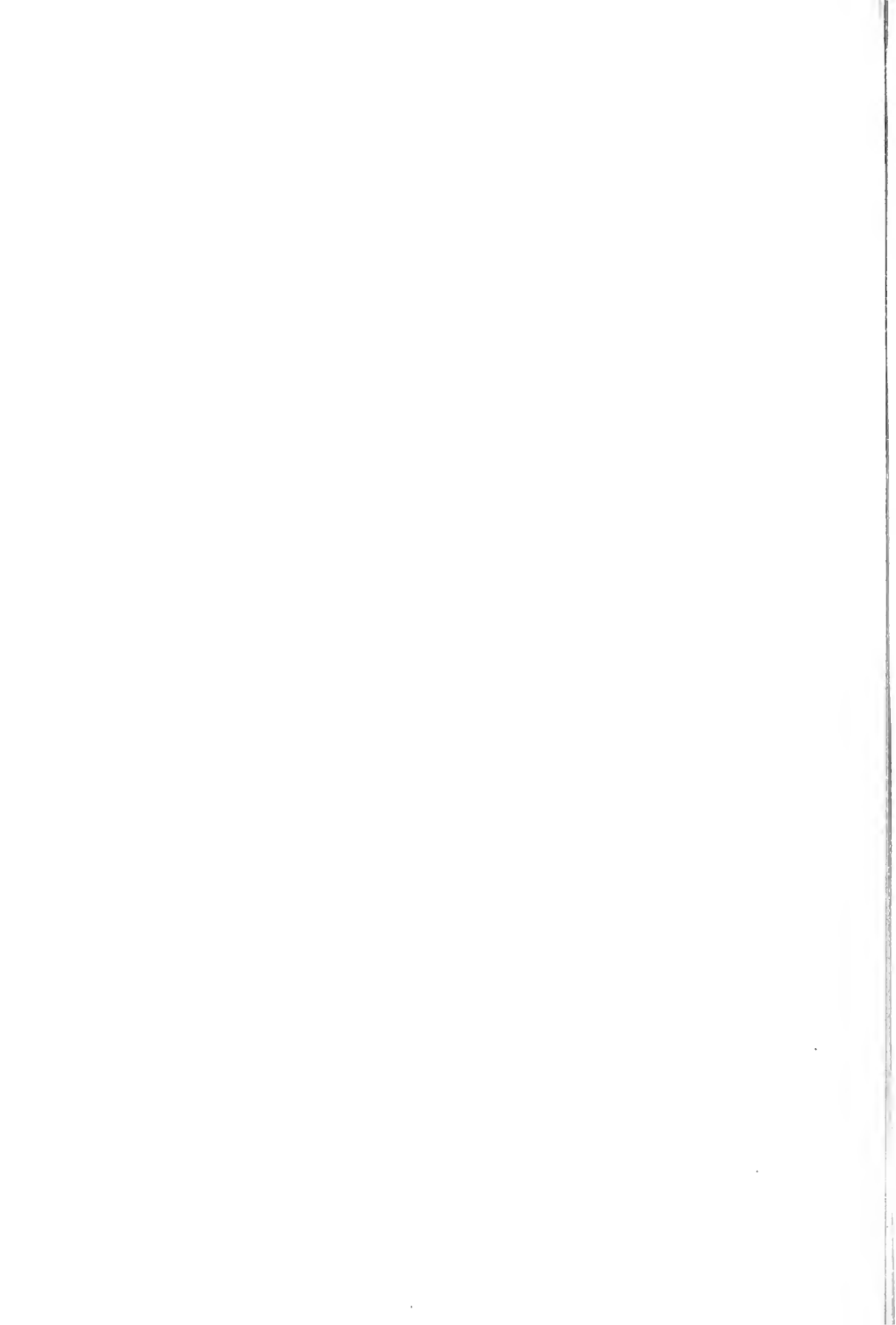
We wish to express our thanks to Dr. J. G. FitzGerald and Dr. R. D. Defries for their helpful criticism and energetic support.

It is a pleasure to acknowledge our indebtedness to Mr. A. S. Wall and Miss Jessie H. Ridout, for their efficient assistance in our work.

⁴ Dudley, H. W., Communicated to Insulin Committee. This method has recently been published (Dudley, H. W., *Biochem. J.*, 1923, xvii, 376).

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COPPER AS A CONSTITUENT IN WOMAN'S AND COW'S MILK. ITS ABSORPTION AND EXCRETION BY THE INFANT.*

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As is well known copper, as well as some of the other common metals, is widely distributed in the tissues of animals and plants. It has been found in various plants and seeds, in egg yolk, in the brain, and many other organs of the body. It is true that the amounts are small, but the very fact that copper is present so constantly has endowed it with particular interest and naturally led to hypotheses as to its function. In egg yolk 20 mg. of copper per kilo of dry matter were found by Fleurent and Levi (1); recently, Bodansky (2) obtained 6.8 mg. of copper per 100 gm. of dry substance from the brain of a 5 month human fetus. In general, it may be stated that copper has been found in greater amounts in animals than in plants, more especially in marine animals.

The analyses of cow's milk have not been numerous. From a consideration of the data of several investigators Bertrand (3) states that cow's milk contains about 0.50 mg. of copper per kilo. The work of Supplee and Bellis (4) gave a similar result—an average of 0.521 mg. of copper for the twenty-three samples tested, irrespective of the ration which the cows consumed. Our interest in this subject followed an investigation by one of us (5) regarding the effect of additions of small amounts of copper to milk on the loss of the antiscorbutic vitamin in the course of pasteurization. A further report of this aspect of the subject will be published in the near future. Quite apart from its relevance to this particular

* Read by title at the meeting of the American Society for the Advancement of Clinical Medicine, May 2, 1923.

question, it seemed worth while to ascertain the copper content of cow's milk after it had been subjected to the process of commercial pasteurization, and to determine the copper content of woman's milk, an aspect of the subject which has not been investigated. Having found copper in these fluids, similar analyses of the urine of infants were carried out, in order to learn whether the copper is absorbed from the alimentary tract or must be regarded merely as an extraneous substance.

The method found most satisfactory for ascertaining small amounts of copper, such as are present in milk, was the ethyl xanthate method recently employed for this purpose by Supplee and Bellis (4). In addition to being highly sensitive, it has the advantage

TABLE I.

Copper in Aqueous Solution as Determined by the Potassium Ethyl Xanthate Method.

Sample No.	Amount of copper present.	Amount of copper found (No. 1).	Amount of copper found (No. 2).
	mg.	mg.	mg.
1	10	10	Not determined.
2	5	5	5
3	1	1	1
4	0.5	0.5	Not determined.
5	0.1	0.1	0.09
6	0.02	0.02	0.015
7	0.002	0.002	Not determined.
8	0.001	0.001	0.001
9	0.0005	Trace or none.	None.

that small quantities of iron, lead, nickel, cobalt, zinc, and manganese do not interfere with its operation. The method is based on the fact that small amounts of copper react with potassium ethyl xanthate to produce a yellow color, and that this color is in direct ratio to the amount of copper present. The reliability of this method is indicated by the data in Tables I and II. The former shows the amount of copper found in 10 cc. volumes of water to which known amounts of copper had been added. Table II shows the amounts of copper recovered from a sample of milk to which definite additions of a copper salt had been made. These determinations indicate that the method may be considered reliable for copper in milk where as little as 0.005 mg. is present in

100 cc. quantities. In our own investigation copper was present always in considerably larger amount.

A sample of raw cow's milk was found to contain 0.55 mg. of Cu per liter. Commercially pasteurized milk contained 0.6, 0.7, and 0.6 mg. in the samples analyzed. It will be noted that these figures correspond fairly well with those published for raw cow's milk, but that the pasteurized milk is somewhat higher. Two samples of woman's milk were analyzed, one from a woman who had an abundance of milk and was able to furnish about 750 cc. daily, of which the copper content was 0.4 mg. per liter; the other from a woman with a less ample secretion of milk who furnished about 460 cc. daily, contained 0.61 mg. per liter. These

TABLE II.

Copper in Milk as Determined by the Potassium Ethyl Xanthate Method.

Sample No.	Amount of copper in 100 gm. milk.	Amount of copper added.	Total amount of copper present.	Amount of copper found.
	mg.	mg.	mg.	mg.
1	0.04	0.02	0.06	0.065
2	0.04	0.005	0.045	0.050
3	0.04	0.005	0.045	0.045
4	0.04	0.004	0.044	0.044
5	0.04	0.003	0.043	0.040
6	0.04	0.002	0.042	0.045
7	0.04	0.002	0.042	0.040
8	0.04	0.001	0.041	0.040
9	0.04	0.001	0.041	0.045
10	0.04	0.0005	0.0405	0.040

women were on a full and varied diet, which included 1 quart of milk a day. As far as can be judged from these analyses, the copper content of the milk of women is about the same as that of the milk of the cow.

It is evident, therefore, that infants, whether breast fed or bottle fed, are regularly consuming small amounts of copper with their food. The next question of interest was whether the copper passes along the gastrointestinal tract as an inert substance, or is absorbed and enters the body fluids. In order to gain information on this point the urine of infants was collected. As it was necessary to obtain at least 2 liters for analysis—the secretion of a week or more—it was not feasible to carry out an exact metabolism test

in all respects. Our main interest, however, lay in the question as to whether copper enters the blood and is distributed to the body tissues. A simple method of ascertaining this fact seemed to be to ascertain whether and to what extent it is regularly present in the urine. Table III summarizes the results of such analyses. It will be noted that in every instance the urine contained copper and that there was approximately one-tenth as much as in woman's or cow's milk. The infants studied were, in all but two instances, 6 to 12 months of age; two were younger. The youngest infant from whom urine was collected was 3 months old, and was on a diet of 600 cc. of milk without the addition of cereal. The urine of this baby contained 0.06 mg. of copper to the liter, and he passed on an average, during the 7 day period of observation, 360 cc. of

TABLE III.
Copper Content of Urine (Mg. per Liter).

Infants.					
Cow's milk and cereal.....	0.066	0.04	0.06*	0.06	0.08
Woman's milk and cereal.....	0.06	0.06			
General diet.....	0.14	0.04	0.65	0.08	0.10
Adults.					
Diet low in Cu.....	0.09	0.08			
“ high in Cu.....	0.14	0.11			

* Received no cereal; 3 months of age.

urine daily, indicating a renal excretion of about 0.02 mg. of copper daily.

The urine was collected from some older infants, 2 to 3 years of age. These were receiving a general diet, composed of milk, vegetables, bread, cereal, sugar, meat, and soup. In two instances, as may be noted in Table III considerably larger quantities of copper were passed than in the younger infants—0.14 and 0.10 mg. per liter. In the former case, the average daily output of urine for the experimental period was 465 cc., indicating a daily urinary excretion of about 0.07 mg. of copper.

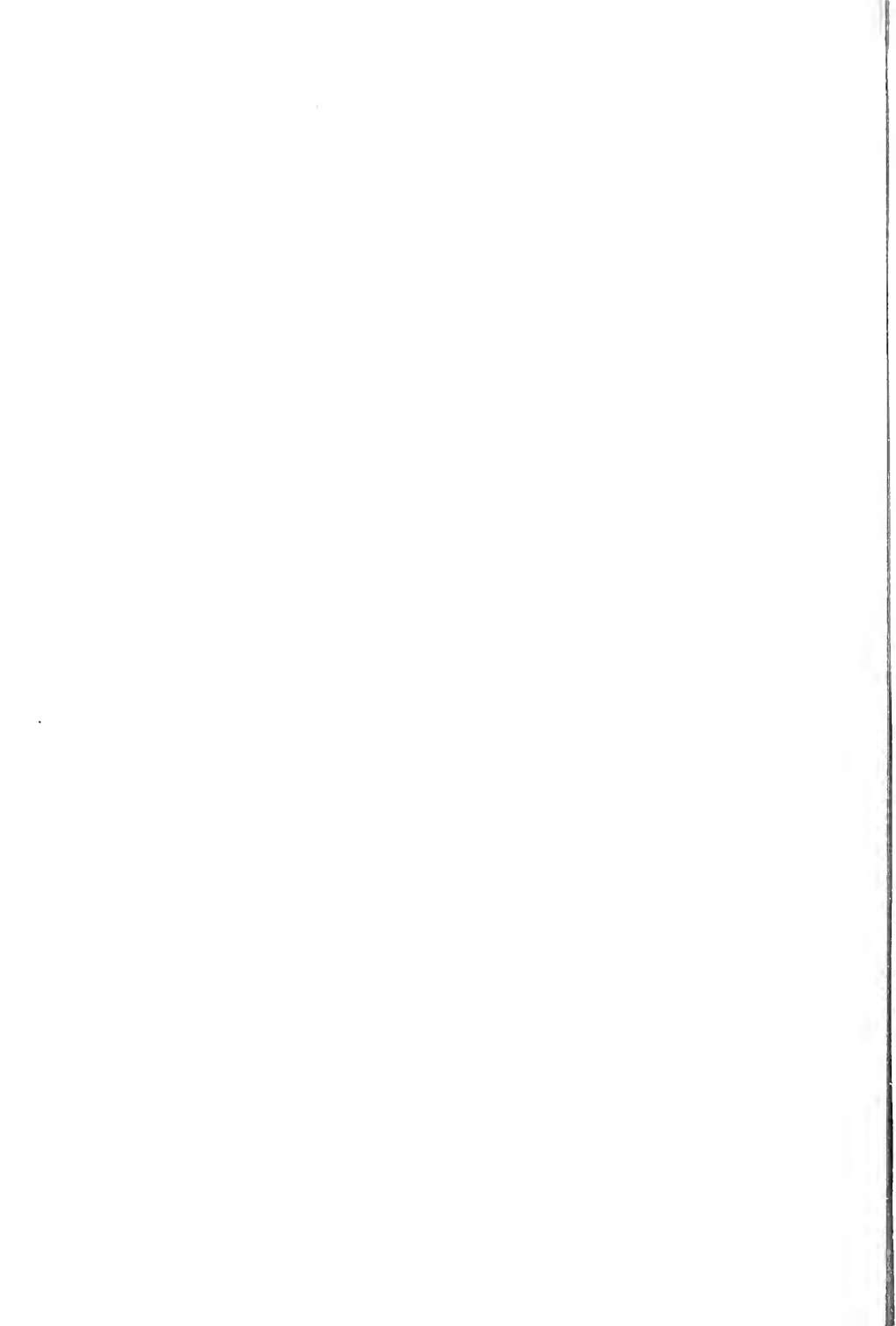
Similar tests were carried out on two adults, one on a diet low in copper and the other on a diet high in copper. The former dietary consisted of milk, corn bread or other corn products,

potatoes, apples, and limited amounts of meat and of fresh spinach. The dietary high in copper consisted of oatmeal with milk or cream, beans, radishes, whole wheat bread or other whole wheat products, pears, lettuce, and a limited amount of potatoes. These diets were maintained for 3 day periods and the urine was collected for the last 36 hours. Between the two test periods there was an interval of 3 days, during which the diet was unrestricted. On the dietary low in copper the urine contained approximately the same amount of copper as was secreted by the younger infants (0.09 and 0.08 mg.), on the dietary high in copper the percentage of urinary copper was higher in both instances.

The fact that copper is not merely present in many of our foods, including woman's milk, but that it is likewise absorbed and incorporated in the body fluids constitutes an additional argument in favor of its exerting some physiological action. It is, however, of little value to speculate regarding this function, or to discuss attractive hypotheses. As is well known, the salts of copper are important catalyzers, so that this form of activity at once suggests itself. Alsberg and Clark (6) expressed the opinion that the copper in hemocyanin acts as a catalyzer for oxygen. In certain instances copper increases the action of the enzyme peroxidase. The question also arises whether it is immaterial if large amounts of copper are absorbed by the tissues—a question which gains added relevancy since the production of experimental hemochromatosis by Mallory, Parker, and Nye (7) by means of chronic copper poisoning. Whatever may prove to be its physiological or pathological significance, our investigations show that cow's milk and woman's milk regularly contain copper, and, furthermore, that in infants and adults copper is absorbed from the alimentary tract as proved by its constant presence in the urine.

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THE INFLUENCE OF THE DIET DURING THE PRE-EXPERIMENTAL PERIOD ON THE SUSCEPTIBILITY OF RATS TO RICKETS.

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In a preliminary communication it was pointed out that "whether or not rickets develops on standard rickets-producing dietaries may depend on the stock of rats which is used," and, furthermore, that "resistance is not to be attributed mainly to peculiarity of strain or breed but to previous diet, for it was overcome by modification of the dietary" (1). These conclusions were drawn from our experience that despite uniformity in all experimental conditions—age, weight, diet of animals, etc.—in some groups of rats rickets regularly developed whereas it did not in others. This seemed a phenomenon of interest and concern for those actively engaged in the study of experimental rickets. It seemed, furthermore, that such inconsistency of results might throw some light on the unaccountable irregularities noted by all clinicians in the development of rickets in infants. With these aspects of the problem in mind, we pursued the investigations further.

In the first instance it was found that rats obtained from one source (Group D, Table I) did not develop rickets when placed on the standard low phosphorus, high calcium dietary (No. 84, Sherman-Pappenheimer). This was our first experience of this kind in the course of an investigation which has included some thousands of rats. In order to gain additional information regarding this lack of conformity, rats of similar age and weight were purchased from several other sources. In no instance, however, did we encounter the same refractory condition, although one other strain developed rickets only to a slight or moderate degree.

Six groups of rats, from six different sources were studied. From Table I it will be seen that they were very similar in weight and age, but that they varied in the content of inorganic phos-

TABLE I.
Inorganic Phosphate of Blood and Susceptibility to Rickets of Various Stocks of Rats.

Stock.	Age.	Weight.	Blood P.	Serum Ca.	Rickets at 8 wks.*	Blood P at 8 wks.
	<i>wks.</i>	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>		<i>mg. per cent</i>
O	4	40	6.8	6.2	Moderate or marked.	2.5
	4	40	7.6			
	4	40	7.6			
	4	40	6.5			
	4	40	6.5			
	4	40	7.5			
G	4	38	9.9	6.1	Moderate or marked.	
S	4	36	7.1	8.2	Moderate or marked.	1.5
	4½	42	7.6			
W	4	40	10.7	7.8	Moderate or marked.	
	4	40	7.7			
	4	50	7.1			
	5	50	8.1			
	5	50				
F	4	40	11.1	6.8	Moderate or slight.	1.5+
	4	40	10.8			
	4½	40	7.8			
	5	44	7.4			
D	4	40	12.0		None.	9.9
	4	30	9.9			7.7†
	4		8.0			

* Sherman-Pappenheimer low phosphorus diet.

† At the end of 15 weeks the inorganic phosphorus was 3.0 mg.; in adult rats of this strain it was 5.7 mg. per cent.

phate of the blood. These blood analyses were carried out when the rats were 4 weeks of age, the period when they were first put on the experimental dietary. For these tests the blood of two or

more rats was pooled, and examined by means of the Bell and Doisy method, as modified by Briggs. These analyses are striking for their marked variation in the inorganic phosphate, which ranges from 6.5 to 12.0 mg. per cent. Such variations do not have their counterpart in analyses of the blood of infants. The nearest approach to this lack of uniformity has been found in the course of examinations of the blood of new-born infants in which, without attributable cause, the inorganic phosphate of the blood was found frequently as low as 3.0 mg. or at times as high as 4.5 mg. per cent (2). It will be noted that the highest percentages were found in Groups D and F, and the lowest in Group O. Although no parallelism was found between the level of inorganic phosphate and the susceptibility to rickets, this factor evidently plays a rôle; for the O group—which had the lowest percentages of inorganic phosphate—developed marked rickets with great regularity, and showed evidences of spontaneous healing least often, whereas the D group—which had the highest percentage of inorganic phosphate—did not develop rickets. Group F stood midway between the two and showed rickets of a mild type with not infrequent radiographic and microscopic signs of healing. Still more striking are the blood analyses when the rats were 8 weeks old and had been on the rickets-producing diets for the regular 4 week period; as may be noted, the inorganic phosphorus showed a marked depletion in all but the resistant stock.

Having failed to induce rickets by means of Diet 84, other dietaries were employed. A diet which we have found serviceable in bringing about rickets is one in which 5 per cent of the flour of No. 84 is replaced by an equivalent percentage of dry milk (dried by the roller process). This food brings about greater growth, as it is more complete, but leads to rickets of milder grade and of somewhat different histological picture. But this dietary likewise failed to induce rickets, except occasionally, in rats of the D group. Some of these animals were placed by Dr. Zucker of this laboratory on more severe rickets-producing diets, containing 10 per cent of casein and various additions of alkalies, without causing rachitic lesions. Furthermore, ten rats were fed a diet high in phosphorus and low in calcium (Diet 85C) but failed to develop rickets; the blood calcium in these animals fell to 4.4 mg. per cent after the test period of 28 days, the inorganic phos-

TABLE II.
Susceptibility of Refractory Rats (Group D) on a Low Phosphorus and a Low Calcium Diet.

Rat No.	Weight. gm.	Rickets-producing diets.	Rickets radiograph (28 days).	Inorganic P, %	Calcium, m. percent	Rickets pathologically.	
						Gross.	Microscopic.
5321	31-60	Low phosphorus (McCollum No. 3143). Whole wheat 33 per cent. " corn 33 " Gelatin 15 " Pure gluten 15 " NaCl 1 " Ca carbonate 3 "	Negative.	7.7	7.4	Very slight.	Negative.
5322	40-70		"			"	"
5323	50-70		"			"	"
5324	40-68		Slight.			"	"
5325	40-72		"			"	Minimal.
5326	40-68		"			Slight.	Slight (atypical).
5327	40-64		"			Very slight.	Doubtful.
5328	30-64		"			"	"
5329	40-70		Negative.			"	Negative.
5330	21-50		Slight.			Slight.	Doubtful (atypical).
5311	30-50	Low calcium (85 C). Flour 95.0 per cent. K ₂ HPO ₄ 2.9 " NaCl 2.0 " Fe citrate 0.1 "	Negative.	9.9	4.4	Very slight.	Negative.
5312	40-54		"			"	"
5313	44-58		"			"	"
5314	34-40		"			"	"
5315	40-44		"			"	"
5316	50-62		"			"	"
5317	42-54		"			"	"
5318	50-60		"			"	"
5319	44-50		"			"	" (osteoporosis).
5320	50-60		"			"	"

* The inorganic phosphate at the beginning of the test was 12.0 mg. (three rats) and 9.9 mg. (four rats) per cent.

phorus being 9.9 mg. per cent. The rickets-producing diet used by McCollum (No. 3143) was next tried. This contains whole grain, gelatin, gluten, sodium chloride, and 3 per cent of calcium carbonate and was fed to ten rats of the standard age and weight. Most of these rats failed to develop rickets, although some showed lesions characterized by Dr. Pappenheimer as atypical (Table II); when killed at 8 weeks of age their blood contained 7.7 mg. per cent of inorganic phosphorus, and the serum, 7.4 mg. per cent of calcium.

It was evident that we were dealing with rats which were highly refractory to diets commonly employed in experimental rickets. The next step was an attempt to break down the refractory state by means of dietetic measures carried out during the preexperimental period—the first 4 weeks of life. The dietary of the Group D rats throughout this period, that is, previous to the time they were under our care, had been exceptionally liberal. Throughout pregnancy and lactation it had consisted of raw meat and bone, milk, whole grain, cabbage, beans, and fruit; egg shells being added during pregnancy. It is difficult to appraise this dietary from a chemical standpoint, but it seems certain that it adequately filled all nutritional requirements. Although we received the rats as soon as they had been weaned, when they were 4 weeks of age, there is no doubt but that, during the latter half of the suckling period, they had consumed some food placed in the cages for the mothers. This factor must be borne in mind; its significance cannot be estimated in regard to the effect of the entire dietary of the lactation period. In order to make a comparison of the protective action of this diet and our standard diet, we obtained some Group D rats at the end of pregnancy and some with litters 1 or 2 days old. These rats were placed on our dietary, which was not as full as that just outlined and consisted of a variety of whole grains, bread, and potato daily; greens twice a week; and meat, cheese, and bananas once a week. Some difficulty was experienced in raising litters, as the mothers, accustomed to a considerable quota of meat, had a tendency to eat their young soon after they were born. However, three satisfactory experiments of this kind were carried out, the details of which may be noted in Table III. For the first test a litter 2 days old was used, for the two others, litters which were born 2 and 4 days, respectively, after the pregnant mother

TABLE III.
Susceptibility of Refractory Rats (Group D) Overcome by Incomplete Dietary Throughout Lactation Period.

Stock.	No. in litter.	Weight. <i>gm.</i>	Diet during pregnancy.*	Diet during lactation period.*	Rickets-producing diets to young.	Rickets in young.		Inorganic P. <i>m. per cent.</i>	Remarks.
						Radiograph.	Microscopic.		
D	9	30-40	Complete.	Incomplete.	No. 84. Dry milk.	Moderate. "	Marked. "	1.5 1.5	Small amount of meat given to mother.
D	5	40-45	"	"	No. 84.	Marked.	"		
D	9	30-40	"	"	" 84.	Moderate.	"	2.09	60 cc. of milk given to mother.
D	6	22-40	Incomplete.	"	" 84.	Marked.			Small amount of brain given to mother.

* See details in text.

had been received by us. All three broods received the standard dietary; one group was given daily 60 cc. of milk in addition, and another a small quantity of meat daily, in order to obviate their tendency to eat the young. As the table indicates, all these rats developed rickets. In these tests the sole factor which had been altered was the diet for the first 4 weeks of life; the surroundings and dietary throughout pregnancy had been the same as that of the mothers of the refractory rats. The young were weaned at 28 days as usual, and were placed on the standard No. 84 diet. In all three tests, comprising twenty-three rats, the rickets was marked as judged by radiographic and microscopic examination. The blood of animals from two of the groups was tested for inorganic phosphate at the time of necropsy and found to be exceedingly low in this constituent—2.09 and 1.5 mg. per cent. When we compare these results with those obtained with the rats which were not reared in our laboratory for the first 4 weeks of life, the interpretation is inevitable that the refractory condition was dependent on the character of the dietary. At present we are unable to state whether the varying susceptibility was due to differences in the milk of the mother or of the solid food which the young consumed during the latter half of the suckling period. Experiments which we carried out recently (1) showed convincingly that, as regards cod liver oil, the active principle is not transmitted through the mother's milk in quantity sufficient to afford significant protection, but is of distinct value when fed directly to the young during the preexperimental period. It is possible that the anti-rachitic factors in food may act differently and be secreted into the milk.

The problem was approached from another standpoint. Rats of Stock D were obtained which were of the same age as those previously tested, but differed in that they had been inadequately suckled. The deficit was brought about by having the dam suckle fifteen to twenty young, instead of merely her own litter which comprises usually five to ten. The dietary of the mother had been the same as that of the previous Stock D rats. As will be noted from Table IV, these animals at the time of weaning were stunted, some weighing only 14 to 16 gm. In every instance rickets developed after they had been on a rickets-producing diet for the period of 4 weeks, whether No. 84 or the dry milk dietary was fed.

TABLE IV.
Susceptibility of Refractory Rats (Group D) Stunted by Inadequate Suckling for First 4 Weeks of Life.

Rat No.	Weight.	Rickets-producing diets.	Rickets radiograph (28 days).	Inorganic P.*	Rickets pathologically.	
					Gross.	Microscopic.
	<i>gm.</i>			<i>mg. per cent</i>		
5305	14-40	Flour 90.0 per cent.	Marked (slight healing?).	3.0	Moderate.	Moderate (slight healing).
5309	30-50	Dry milk . . . 5.0 "	Moderate.		"	"
5310	24-50	Ca lactate . . . 2.9 "	Marked.		Marked.	Marked.
5301	20-40	NaCl 2.0 "	"	1.5+	Moderate.	Slight.
5302	30-50	Fe citrate . . . 0.1 "	Moderate (slight healing).		"	Moderate (slight healing).
5304	24-40		"		"	Marked.
5308	14-38		Marked.	2.0	Slight.	Slight.
5291	28-40	No. 84.	Slight.		Marked.	Moderate.
5293	16-30	Flour 95.0 per cent.	Moderate.		Slight.	" (slight healing).
5294	20-32	Ca lactate, 2.9 "	Slight.			
		NaCl 2.0 "				
		Fe citrate . . 0.1 "				

* The inorganic phosphate of the blood of these stunted rats was 7.1 mg. per cent at beginning of the test.

After this interval the inorganic phosphate of the blood became unusually low, from 1.5 to 3 mg. per cent. It would seem that the difference in susceptibility between the normal and the stunted rats of this stock is attributable to the fact that the latter were unable to store protective factors during the suckling period, so that they were potentially rachitic. No doubt the rapid gains which ensued when they were placed on the experimental dietaries—some even doubled their initial weight—greatly tended to the development of rickets. The nutritional condition calls to mind that of the infant who has been nursed inadequately—due to an insufficient supply of milk or to unduly prolonged nursing—and is later given a bountiful diet. A sharp gain in weight ensues, accompanied by a marked tendency to the development of rickets.

It is evident that we are dealing with a marked lack of susceptibility to rickets, mobile in character and readily overcome by alteration of dietary. This condition seems best accounted for on the theory that, during the preexperimental period, the first 4 weeks of life, the rats stored a protective substance which they had obtained either indirectly through the mother's milk or directly from other food. If we accept the explanation of protection through the agency of mother's milk, the physiological mechanism must differ from that governing cod liver oil, which was found ineffective in attempts to supply its active principle to the young indirectly through this medium (3). This question must be left open as young rats after the 10th day of life frequently partake of the food provided for the mother. However this may have been, our experience indicates that in experiments on rickets, especially those of a quantitative or comparative nature, the dietary must be not only standardized throughout the experiment, but must be controlled likewise during the preexperimental period. In a recent study of the fat-soluble vitamin Steenbock and Nelson (4) have drawn attention to the necessity of considering "the vitamin reserves" in the study of growth or incidence of pathological conditions. Korenehevsky (5) has stressed the importance of the fat-soluble vitamin and calcium in the diet of the lactating mother, and is of the opinion that the young may be protected against rickets by obtaining a liberal amount of these factors through the mother's milk. Although we agree as to the importance of diet throughout the lactating period, its antirachitic value,

in our opinion, does not depend on its content of either of these constituents. This question, however, as well as that of the rôle of diet during the prenatal period, will be considered in connection with experiments to be reported later.

SUMMARY.

Rats may fail to develop rickets on the standard "rickets-producing dietaries" whether low in phosphorus or in calcium. This term cannot, therefore, be applied unreservedly to experimental diets.

The refractory state was found to be due to the liberality of the diet during the preexperimental period, the first 4 weeks of life.

It was overcome and the young were rendered susceptible to rickets by feeding mother and young a less adequate dietary throughout the suckling period.

Resistance was also broken down by means of inadequate lactation during this period, by having the mother suckle young in addition to her own litter. This deprivation probably resulted in an inability on the part of the young to store a reserve of the antirachitic factor.

Experiences of this kind indicate the necessity of controlling the diet of experimental animals for the entire period preceding the test. They also suggest that the diet of infants during the first weeks of life may be of equal importance in relation to the later development of rickets.

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ETHER ANESTHESIA.

I. THE DETERMINATION OF ETHYL ETHER IN AIR AND IN BLOOD, AND ITS DISTRIBUTION RATIO BETWEEN BLOOD AND AIR.*

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On the hypothesis that the passage into the blood of ether and other volatile substances used for inhalation anesthesia is a process of simple diffusion of the gas from the alveoli in accordance with the distribution coefficient of the substance between air and blood at the body temperature, and on the further hypothesis that the production and maintenance of a given degree of anesthesia depends upon a certain definite concentration of the anesthetic in the blood and tissue fluids bathing the nerve cells or other elements, it should be possible to establish the following values and relationships. (1) The anesthetic concentration in the blood, (2) the concentration of the anesthetic gas in the air breathed necessary to maintain the anesthetic concentration in the blood, and (3) the ratio between these concentrations in blood and alveolar air should be constant, and *should have the same numerical value as the distribution ratio for the anesthetic between blood and air, at body temperature, in vitro.*

The determination of these quantities would decide the correctness of the hypothesis and would be of practical as well as theoretical interest.

On consulting the literature we were surprised to find great discrepancies between the values obtained by different investigators for, on the one hand, the anesthetic tension of ether in the air breathed, and on the other hand, the concentration of ether

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in the blood during anesthesia. The ratio between the two had not been accurately determined either *in vitro* or *in vivo*.¹

The underlying principles of the absorption of inhalation anesthetics were first investigated and clearly stated by John Snow (2) in 1848, immediately after the demonstration of the anesthetic properties of ether and chloroform. Snow performed a series of notable experiments from which he arrived at the concentration of chloroform, ether, and other vapors required to induce and maintain different degrees of narcosis, and by calculation he estimated the amount of the anesthetic present in blood during anesthesia. He very definitely expressed the idea of the relation between concentration of vapor inhaled and concentration in blood at the body temperature as determining the depth of anesthesia.

For a mouse at 100°F. Snow found 1.75 volumes per cent of ether vapor "sufficient to induce the second degree of narcotism," and 3.5 volumes per cent, the "fourth degree of narcotism." He calculated that the blood contained in these two conditions 0.0875 per cent and 0.175 per cent of ether, respectively. For birds with higher body temperatures the concentration of vapor producing narcosis was higher and for frogs much lower. Since Snow's time it appears to have been supposed that the concentration of ether vapor necessary to maintain anesthesia should be a fairly constant value, but the results obtained by different workers vary from 2.1 to 10.6 volumes per cent in the air breathed, and from such variations it is impossible to conclude whether the anesthetic tension is actually constant, or what its value is.

The concentration of ether in blood has been directly determined by only two investigators (3, 4), but their results are in substantial agreement, and the work of Nieloux (3) indicates that there is only a small margin between the concentrations which produce anesthesia (0.105 to 0.110 per cent) and those which

¹Very recently Haggard (1) has determined the distribution ratio of ether between water and air, and blood and air, *in vitro*, his work being prompted by the same considerations which led to our investigation. The work reported in this paper was completed before the appearance of Haggard's communication. At body temperature our results are in satisfactory agreement with his.

stop respiration and are fatal (0.160 per cent). But if the anesthetic concentration of blood is constant, and only slightly below the fatal concentration, an increase of tension of ether in the air breathed, from 3 to 10 volumes per cent, would raise the amount in the blood to three times its initial value, or much above the fatal concentration. The results are therefore contradictory, and the subject requires further study.

In the fall of 1921 we undertook the determination of the ratio of distribution of ether between water and air and between blood and air, at different temperatures, in order to learn whether during ether inhalation anesthesia the same ratio exists between ether in the blood and in the alveolar air. The demonstration of this relationship would prove the absorption of ether to be the result of diffusion, and would clarify existing doubt as to the anesthetic tension of ether in air breathed. Our results show that the expectation is correct, at least in dogs, the ratio of ether in blood to ether in alveolar air during anesthesia being substantially the same as the distribution ratio *in vitro* at the same temperature.

We are therefore able to prove the simple relationships pointed out by Snow three-quarters of a century ago but since obscured by the conflicting data of later investigators. It may be added that although our results differ from those of a number of recent workers, they are in remarkable agreement with the values found by Snow.

It became apparent at the outset that the main cause of discrepancies in results from past work is the inadequacy of the analytical methods used for the determination of ether. Our first attempt was, therefore, to develop a method so that more reliable results might be obtained. It is proposed in this paper to describe the method of determination, and to report the values obtained for the distribution ratio between water and air and blood and air *in vitro*. In the following paper (5) will be given the data for animal experiments, and we shall later report observations during ether anesthesia on human subjects.

Methods based upon the oxidation with mixtures of sulfuric acid and bichromate have been frequently used for the determination of alcohol, and are equally applicable to ether, and appeared to be the most promising for our purposes. Both

alcohol and ether form stable ethyl sulfuric acid, which under certain conditions is quantitatively oxidized by chromic acid to acetic acid. In various forms this reaction has been used for alcohol by Bodländer (6), Frankland and Frew (7), Dupré (8), Benedict and Norris (9), Nicloux (3), Dox and Lamb (10), Thorpe and Holmes (11), Pringsheim (12), Widmark (13), and Miles (14), and for ether by Nicloux (3) and van Leeuwen (4).

Nicloux removed the ether from water solutions by aeration, the air being passed through 50 per cent sulfuric acid, which absorbs the ether with formation of ethyl sulfuric acid. By subsequent addition of measured amounts of standard bichromate, the ether is oxidized to acetic acid, the end-point being indicated by the appearance of a yellowish tinge in the green solution. In our hands this method worked poorly, chiefly due to difficulty in seeing the end-point, which is indistinct. But it was found that the use of an excess of bichromate for the oxidation and the subsequent determination of the excess by iodometric titration converts the Nicloux procedure into an exceedingly simple, delicate, and satisfactory method.

After the oxidation is completed, the acid is rinsed from the large test-tubes in which the aeration and absorption of ether take place, is diluted with 100 to 200 cc. of water, an excess (2 to 4 gm.) of KI is added, and the liberated iodine is titrated with standard thiosulfate and starch. A blank titration is made with the same amount of bichromate solution and acid, the difference representing the chromic acid used for the oxidation of the ether. Each molecule of ether required 4 atoms of oxygen, and 1 cc. of 0.1 *N* iodine is therefore equivalent to 0.926 mg. of ether.

The accuracy of the method based on the above reactions requires that the oxidation to acetic acid be quantitative, and that no further oxidation of acetic acid take place. Our experience has shown that these requirements are met only under certain conditions. The use of concentrated sulfuric acid favors completeness of absorption of ether vapor, and increases the rate of oxidation, and at room temperature there is little or no further oxidation of acetic acid. But at higher temperatures with concentrated acid some acetic acid is oxidized, and the amount of bichromate reduced is correspondingly great. Even the rise of

temperature resulting from dilution of the concentrated acid by the addition of bichromate solution or of water may lead to results which are a few per cent too high. That these high results are due to oxidation of acetic acid to CO_2 was indicated by passing the air, after removing the ether in concentrated sulfuric acid bichromate mixture, through barium hydroxide solution, barium carbonate being precipitated.

This observation led us to test directly the oxidation of acetic acid by sulfuric acid and bichromate. Pure acetic acid was distilled from sulfuric acid and bichromate. The material thus prepared did not reduce chromic acid in concentrated H_2SO_4 in the cold, but there was some reduction when heated to boiling, as shown in Table I.

TABLE I.

Oxidation of 5 Cc. of Approximately N Acetic Acid by Concentrated H_2SO_4 + Bichromate.

Condition.	27°C.*	10°C.	Ice box.*	27°C.*	Heated 20 min. Remainder 27°C.
Allowed to stand, hrs.....	1	1	24	24	24
Amount of bichromate reduced, cc. of 0.1 N.....	0.1 0.0 0.05	14.0 12.3 11.8	0.1 0.0 0.0	0.0 0.0	9.0 11.4

* The tubes were kept cold while mixing the H_2SO_4 and acetic acid solution and during dilution with water preliminary to titration.

It will be noted that at temperatures below 27°C. the acetic acid remained unoxidized in the presence of the sulfuric acid bichromate mixture even after 24 hours.

During the process of aeration the tubes of sulfuric bichromate mixture in which the ether is collected become hot, due partly to the heat of reaction, and partly to the heat generated by the absorption of water. This initial high temperature was thought to be the cause of the formation of small amounts of CO_2 observed. The following experiment was conducted, keeping the temperature of the acid low by placing the tubes in an ice and salt bath.

After passing through the acid the air was tested for CO_2 by passage through a tube containing $\text{Ba}(\text{OH})_2$ solution. Another set of determinations was made, identical except that there was no control of temperature of the chromic acid mixture. The results are given in Table II.

TABLE II.

Production of CO_2 by Oxidation of 131 Mg. of Ether by Aeration into Concentrated H_2SO_4 + Bichromate.

No.		Period of aera- tion.	0.1 N thio- sulfate used.	Ether found.		BaCO_3
		min.	cc.	mg.	per cent	
1	Blank, no bichromate.	20				Trace.
2	" " ether.	20	152.1			"
	" " "	60	152.1			None.
3	Ether, bichromate at 0°C .	20	61.1	84.2	64.3	Trace.
4	" " " no tem- perature control.	20	5.1	136.0	103.8	+++

It is evident from this experiment that lowering the temperature stops the formation of CO_2 , but also greatly retards the oxidation to acetic acid, only 64.5 per cent of the ether being oxidized in the tube kept at 0°C .

By allowing sufficient time at room temperature, the oxidation of ether to acetic acid is complete, and by preventing the initial rise in temperature during collection of the ether the formation of CO_2 may be avoided. Into each of a series of large test-tubes there were measured 10 cc. of a water solution containing by weight 20.8 mg. of ether. Each tube was aerated for 20 minutes into the same amounts of sulfuric bichromate solution, the acid tubes being kept in an ice and salt bath. The tubes were then removed and brought at once to room temperature by placing in warm water, and were diluted and titrated after varying intervals of time. The results are shown in Table III. It appears from these data that ether may be completely oxidized to acetic acid without the formation of CO_2 if collected cold and allowed to stand for 3 hours at room temperature. It was found, however, that even though kept cool during the process of collection the sudden rise in temperature on washing from the test-

tube and dilution for titration, caused further oxidation. This could be avoided by conducting the dilution slowly under a stream of cold water. High results were more frequently noticed when large quantities of ether were oxidized. To avoid errors arising from this source, collection was conducted in concentrated sulfuric acid which was diluted to 40 per cent and cooled before the addition of bichromate. The mixture was then heated in a

TABLE III.

Time Required for Oxidation of Ether at Room Temperature in Concentrated H_2SO_4 .

Aeration into cooled concentrated sulfuric chromic acid.

Time.	Thiosulfate used.	Ether found.	Ether.
		<i>m.g.</i>	<i>per cent</i>
10 min.	18.5	17.6	84.4
20 "	18.7	17.8	85.4
30 "	19.3	18.4	88.4
50 "	21.3	20.3	97.5
60 "	21.0	20.0	96.1
70 "	20.8	19.8	95.2
1 hr. 30 min.	21.3	20.3	97.5
1 " 30 "	21.4	20.4	98.0
2 hrs.	21.6	20.5	98.5
2 "	21.6	20.5	98.5
3 "	21.7	20.7	99.5
4 "	22.5	21.4	102.8
4 "	21.8	20.8	100.0
24 "	21.7	20.7	99.5
24 "	21.7	20.7	99.5
12 " ice box.	19.3	18.4	88.4

water bath to just below boiling for 30 minutes. With these modifications the results are accurate to within ± 2 per cent.

Nieloux appears not to have had the difficulty of partial oxidation to CO_2 . When 50 per cent sulfuric acid is used for collection the oxidation evidently stops with the formation of acetic acid. But with acid of this strength boiling is necessary to complete the oxidation to acetic acid. Table IV is a protocol of an experiment to test this point.

TABLE IV.

Effect of Heating Ether in 50 Per Cent Sulfuric Acid Bichromate Mixture.

10 cc. portions of solution containing 19.4 mg. of ether were aerated through three tubes, each containing 10 cc. of 50 per cent H_2SO_4 solution of bichromate.

No.	Ether de- termined.	Ether recovered.	Conditions of experiment.
	mg.	per cent	
1	16.0	82.5	Titrated 20 min. after aeration at room temperature.
2	18.9	97.5	Allowed to stand in water bath at 100° 30 min. before titration.
3	19.45	100.3	" " "
4	19.52	100.7	Heated to boiling over free flame for 5 min. Allowed to stand 30 min. in boiling water before titration.
5	19.43	100.2	Heated to boiling, allowed to cool, and heated again. Allowed to stand 30 min., then titrated.
6	19.35	99.7	Boiled for 3 min. over free flame and titrated when cool.

A known amount of ether, 19.4 mg., was aerated into tubes containing a solution of potassium bichromate in 50 per cent sulfuric acid. Three tubes were used in series to insure complete collection. Between 3 and 5 per cent of the total ether was found in the second tube of the series, and with a single exception a trace was always found in the third. One may therefore collect the ether in at least three tubes of 50 per cent sulfuric acid containing bichromate and heat each to boiling to complete the oxidation; or absorb the ether in one tube of concentrated acid, subsequently dilute it with water to 40 or 50 per cent, add a carefully measured amount of bichromate solution, and heat to boiling or in a boiling water bath for 30 minutes. In either case the solution is finally cooled, rinsed into a flask, and diluted before adding the iodide. The end-points of the titration are sharp and definite. We have more frequently used the second procedure stated above.

The accuracy of the results in the tables is based upon weighed amounts of pure ether dissolved in water, in air, and in blood. The ether used was especially purified for the purpose and care-

fully tested by the laboratory of the Mallinckrodt Chemical Company. It gave no test for aldehyde, peroxide, alcohol, or acetic acid.

The method of preparing and of measuring known solutions of ether was the following. Samples of the ether were sealed in tared glass bulbs and weighed. These were dropped in long necked

TABLE V.
Determination of Ether in Water.

Aeration into one tube of concentrated H_2SO_4 (see p. 748).

No.	Amount of solution.	Amount of ether in sample.		Variation from known.
		By weight.	By analysis.	
	cc.	mg.	mg.	per cent
1	10	30.24	30.12	-0.4
2	10	30.24	30.12	-0.4
3	10	30.24	30.70	+1.52
4	5	15.12	14.81	-2.05
5	5	15.12	15.32	+1.32
6	5	15.12	15.01	-0.73
7	5	8.44	8.30	-1.66
8	5	8.44	8.35	-1.07
9	5	8.44	8.42	-0.24
10	5	5.68	5.78	+1.76
11	5	5.68	5.80	+2.11
12	5	5.68	5.71	+0.53
13	10	15.55	15.25	-1.93
14	10	15.55	15.65	+0.64
15	10	15.55	15.32	-1.48
16	10	15.55	15.67	+0.77
17	5	3.85	3.81	-1.04
18	5	3.85	3.92	+1.82
19	5	3.85	3.80	-1.30
20	5	3.85	3.88	+0.78
Maximum error, per cent.				± 2.11

flasks nearly filled with water (or blood) and broken by shaking against the side. The ether quickly dissolved in the large excess of liquid. The long neck of the flasks prevented appreciable diffusion into the air while samples were being measured. Pipettes used for measuring were calibrated between two points on the stem so that the part of the solution in contact with the air could be rejected. Table V is a tabulation of the results obtained with

solutions of ether in water by the second procedure described above. The maximum error is ± 2 per cent.

Determinations of ether in blood are complicated by foaming. Volatile antifoaming substances reduce bichromate so it is necessary to carry on the aeration without their use. Since the distribution coefficient of ether for blood and air is low it has been found that by using a flask with a large surface over which the blood is spread and aerating over the surface, the ether is entirely removed in a remarkably short time. 90 per cent is removed in the first 30 minutes and in from $1\frac{1}{2}$ to 2 hours every

TABLE VI.
Ether in Blood.

Ether was weighed in a thin glass ampule, placed in a flask containing blood, and broken by shaking against the wall of the flask.

No.	Amount of blood used for analysis.	Amount of ether in sample.		Variation from known.
		By weight.	By analysis.	
	cc.	mg.	mg	per cent
1	2	1.862	1.872	+0.54
2	2	1.862	1.841	-1.13
3	3	2.793	2.753	-1.43
4	3	2.793	2.786	-0.25
5	5	4.655	4.655	0.0
6	5	4.655	4.621	-0.73
7	2	4.322	4.296	-0.60
8	2	4.322	4.372	+1.17
9	5	10.805	10.516	-2.67
10	5	10.805	10.913	+1.00
Maximum error, per cent.....				± 2.68

trace is removed. Oxalate should be added to prevent clotting and if added in excess tends to make the ether less soluble in blood and so facilitates its removal by aeration. Warming the flasks and occasional rotation greatly hasten the process.

Aeration of a sample of blood containing no ether showed no volatile substances.² Determinations on blood are given in Table VI. The maximum error is ± 2.68 per cent.

² Alcohol if present would be aerated into the sulfuric acid and oxidized. The distribution ratio of alcohol at room temperature is, however, so

The determination of ether in air was accomplished in the following way. From the partial pressure of ether in air over pure ether, which is known (15) for various temperatures, and assuming ether to follow the gas laws, the amount of ether in a sample of such air was estimated. Samples of this ether-air mixture were measured over mercury, diluted with a measured volume of air, and forced out of the burette slowly through sulfuric acid which collected the ether. Determinations were completed in the usual way. Dry air was used and the whole proce-

TABLE VII.
Ether in Air.

Air containing by calculation 80.5 mg. per 100 cc. was drawn into a sampling tube over mercury and diluted to ten times its volume so that 100 cc. contained 8.05 mg. of ether. Measured amounts of this air were analyzed.

No.	Amount of air.	Amount of ether in sample.		Variation from known.
		Calculated.	Determined.	
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	50	4.025	3.955	-1.74
2	50	4.025	4.027	+0.05
3	100	8.05	8.211	+2.00
4	100	8.05	8.002	-0.60
5	200	16.10	16.338	+1.48
6	200	16.10	16.001	-0.61
7	300	24.15	24.39	+0.99
8	300	24.15	23.440	-1.20
Maximum error, <i>per cent.</i>				±2.00

dure done at room temperature. The results obtained (Table VII) indicate also that ether vapor conforms to the gas laws at the temperatures used. The maximum error in the determinations was ± 2.0 per cent.

different from ether that the two may be roughly separated by aeration. If both are present in about equal amounts a period of aeration which completely removes the ether carries over only 5 or 10 per cent of the alcohol. Acetone is not oxidized under the conditions of the determination and does not interfere.

Distribution Ratio of Ether between Water and Air.

For equilibrating the air and ether solutions the method employed by Briggs and Shaffer (16) for determining the distribution coefficient of acetone in an air-water system was employed. A series of five bottles containing the same concentration of ether was placed in a thermostat. A thermometer in the last bottle of the series made it possible to register the temperature at that part of the system where the air was in equilibrium with the solution. This bottle was also connected with a water manometer so that the pressure within the system when the air was flowing could be kept at atmospheric pressure. This was done by applying just enough suction at the outlet to balance the pressure of the inlet. Description and diagram of this apparatus are given in the paper referred to (16).

The ether-air mixture was passed through H_2SO_4 removing the ether which was determined as already described. The volume of air remaining was measured by displacement of water. The sum of the volumes of air and ether vapor corrected to the temperature and pressure of the final equilibrating bottle, moist, gives the volume of gas containing the ether. The ratio between the concentration in air and in the solution is the distribution ratio at the observed temperature.

Before and after each sample of air was measured samples of the solution were taken by means of a pipette which fitted onto a stop-cock. The pipette was calibrated to read between two points so the top layer in contact with the air was always rejected.

This proved to be a very practical method since the air in passing through the first four bottles which contained large quantities of the solution came to equilibrium with regard to the ether before it reached the final bottle from which the samples were made. The loss of ether from this bottle was inappreciable over long periods of time, making it possible to do a series of experiments at different temperatures on the same solution. As soon as a falling off in the ether content of the sample bottle was noted the solutions were renewed.

Example of Calculation.

The temperature of the water solution of ether in equilibrium with air was 37.6°C. and the barometer 760 mm. 500 cc. of air, measured at a temperature of 30.4°C. after ether had been removed, were found to have contained 26.60 mg. of ether. The gases in equilibrium with solution and the air as measured are both saturated with water. The solution in the last equilibrating bottle was found by analysis to contain 773 mg. of ether per liter.

Assuming ether vapor to behave as a perfect gas, a gram-molecule, 74.1 gm. = 22.4 liters at 0°C. and 760 mm. pressure, or 1 mg. = 0.3022 cc. When moist, at 37.6°C. and 711.8 mm. pressure (760 - vapor tension of H₂O at 37.6°), the conditions of equilibration,

$$1 \text{ mg. ether} = 0.3022 \times \frac{273 + 37.6}{273} \times \frac{760}{760 - 48.2} = 0.3671 \text{ cc.}$$

and 26.6 mg. ether occupied

$$26.6 \times 0.3671 = 9.765 \text{ cc.}$$

The 500 cc. of air measured at 30.4°C., occupied at 37.6°C., moist,

$$500 \times \frac{273 + 37.6}{273 + 30.4} \times \frac{760 - 32.3}{760 - 48.2} = 523.3 \text{ cc.}$$

And the total volume of moist air and ether vapor was 523.3 + 9.76 = 533.06 which contained at 37.6°C., 26.60 mg. of ether or 49.9 mg. per liter.

$$\frac{773}{49.9} = 15.5 \text{ distribution ratio at } 37.6^\circ\text{C.}$$

Table VIII shows the distribution of ether in a water-air system at different temperatures and concentrations.

The distribution ratio for blood was determined on defibrinated beef blood, dog blood, and human blood. When beef blood was used the method of equilibration was the same as for water. With dog and human blood equilibration was accomplished in a separatory funnel in a thermostat. The air was measured in the funnel and forced out by mercury into H₂SO₄ where the ether was collected and later analyzed. A sample of blood was taken by means of a pipette and analyzed at once. Table IX shows the determinations obtained on blood.

The curves constructed from these data are given in Chart 1 together with a curve based on the partial pressure of ether in an air-water system at saturation. Between 35 and 40° the data here presented check with those recently reported by Haggard (1). Below 35° our ratios are somewhat higher.

TABLE VIII.

Distribution Ratios for Ether in a Water-Air System.

No.	Temperature.	Amount in 1 liter solution.	Amount in 1 liter air.	Amount in solution. Ratio Amount in air
	°C.	mg.	mg.	
1	12.2	362.0	6.82	53.1
2	12.2	362.0	6.84	53.0
3	12.2	362.0	6.81	53.2
10	12.1	2,200.0	41.50	53.0
11	12.15	2,200.0	41.70	52.8
12	12.2	2,200.0	41.40	53.2
4	16.0	362.0	7.72	46.9
5	16.0	362.0	7.69	47.1
6	16.0	362.0	7.70	47.0
13	16.3	2,200.0	46.80	47.0
14	16.3	2,200.0	47.20	46.7
15	16.3	2,200.0	47.20	46.7
16	18.2	2,200.0	51.20	43.0
17	18.2	2,200.0	51.00	43.2
6	20.0	362.0	9.16	39.5
7	20.5	362.0	9.05	40.0
8	20.2	362.0	9.29	39.0
9	25.8	362.0	12.12	29.8
18	28.0	780.0	29.80	26.2
19	31.0	780.0	36.30	21.5
23	31.0	780.0	36.20	21.5
24	33.5	672.0	34.85	19.3
25	33.0	672.0	34.65	19.4
20	35.5	780.0	46.40	16.8
26	36.6	350.0	21.60	16.2
27	37.5	350.0	22.16	15.8
31	37.6	773.0	49.90	15.5
28	38.5	350.0	23.30	15.0
29	39.0	381.0	26.80	14.2
21	40.0	780.0	55.60	14.0
22	40.0	780.0	55.60	14.0
30	40.0	420.0	29.90	14.0

As a check on the theoretical curve (Chart 1) and therefore on the measured curves, since these agree fairly closely, the

TABLE IX.
Distribution Ratios for Ether in a Blood-Air System.

Kind of blood (defibrinated).	Temperature.	Amount in 160 cc. blood.	Amount in 100 cc. air.	Ratio $\frac{\text{Amount in blood}}{\text{Amount in air}}$
	$^{\circ}\text{C}.$	m_j .	m_j .	
Beef.	20.0	120.2	3.49	34.5
	25.3	120.2	4.52	26.6
	27.0	120.2	4.85	24.8
	28.5	120.2	5.36	22.4
	35.0	120.2	7.51	16.0
	37.0	120.2	8.05	14.9
	39.0	120.2	8.89	13.5
	39.2	87.9	6.49	13.5
	38.0	87.9	6.27	14.0
	37.2	87.9	6.15	14.3
	36.5	87.9	5.98	14.7
	32.0	87.9	4.78	18.4
	30.0	87.9	4.33	20.3
	23.5	87.9	2.97	29.6
Human.	37.5	123.62	8.34	14.8
	37.0	123.4	8.27	14.9
	37.0	97.23	6.45	15.0
	37.0	84.61	5.72	14.7
	36.5	156.34	10.31	15.2
	36.8	58.42	3.82	15.3
Dog.	39.0	216.21	15.65	13.8
	39.3	132.81	9.78	13.6
	38.9	103.5	7.60	13.6
	39.2	109.6	8.37	13.1
	39.0	74.2	5.50	13.5

vapor pressure over mutually saturated solutions of ether + water was determined. The total vapor pressure is composed

of two components, water and ether vapor. The partial pressure of ether over this solution should be less than that over pure ether by an amount equal to the molecular lowering due to water

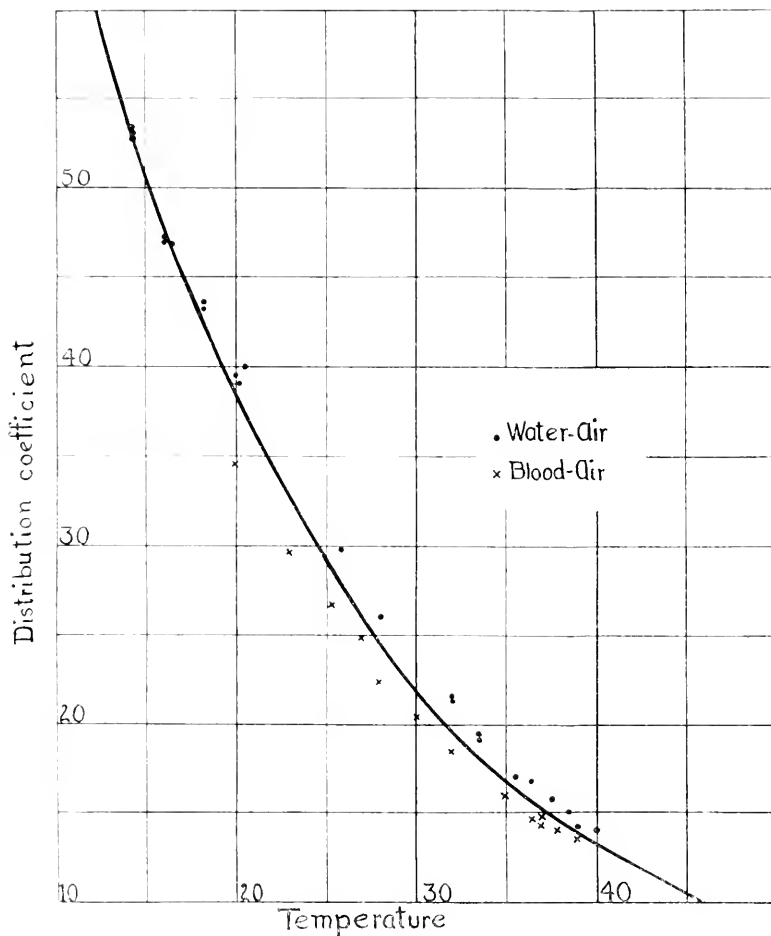


CHART 1. Distribution curve of ether in an air-water system calculated from the determined partial pressure of ether at saturation for different temperatures.

● represents observed distribution coefficients of dilute solutions of ether in an air-water system.

× represents distribution coefficients observed in an air-blood system.

dissolved in the ether layer. In so far as water and ether form ideal solutions, this would be proportional to the concentration of the water in the ether layer. Thus the partial pressure of ether over saturated solutions as calculated from the concentration and the partition coefficients for dilute solutions should agree with measured values of vapor pressure over mutually saturated solutions of ether and water minus the vapor pressure of water. The molecular lowering of the water vapor pressure due to the presence of ether is negligible as demonstrated later. Chart 2 gives the measured (Curve 2) and computed values (crosses). That these do agree is a check on the measurements of the partition coefficients.

The determination of the vapor pressure over saturated solutions was measured by enclosing a solution of ether and water with an excess of ether in a separatory funnel connected with a mercury manometer. The air was carefully boiled off, the whole apparatus brought to the desired temperature in a thermostat, and the pressure read at different temperatures. The mixture was agitated by a splasher kept in motion by means of an electromagnet on the outside of the glass separatory funnel, the splasher forming the armature, the current being interrupted by a pendulum operated magnetically in the same circuit.

On Chart 2 is also drawn a theoretical curve (Curve 5) for the molecular lowering of the ether partial pressure due to the presence of water. This is computed from the formula for thermodynamical molecular lowering (17). The reason for the observed lowering being less than the expected is not explained and is being further investigated. Whether this is due to polymerization or to adsorption and surface activity, dilute solutions would be expected more nearly to approach the theoretical curve than those observed for saturated solutions. The lowering of the vapor pressure would be proportionally greater at saturation and the distribution ratios corresponding to those values would be even higher than those computed at saturation and not lower, as indicated by Haggard's results (1).

As a check on the accuracy of these curves, vapor from a mutually saturated solution of ether and water was estimated by determination of the saturation temperature of the ether; that is, the temperature at which the ether became cloudy due to

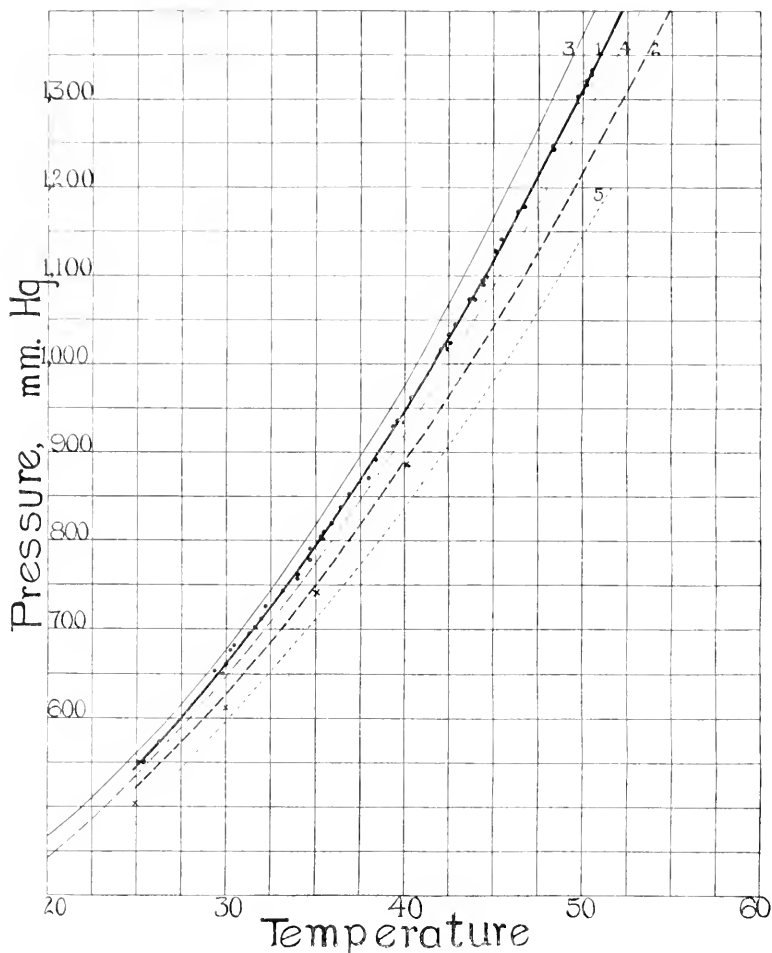


CHART 2. Curve 1, observed vapor pressure over mutually saturated ether-water mixture at different temperatures. Curve 2, Curve 1 minus the vapor tension of water at temperature observed. Represents the partial pressure of ether. Curve 3, vapor pressure of ether over ether at different temperatures plus the vapor tension of water at the same temperature. The space between Curves 1 and 3 represents the lowering of the vapor tension due to dissolved substances. Since the lowering of the water vapor tension is negligible this amount is equal to the lowering of the vapor tension of ether. Curve 4, vapor pressure of ether over ether at different temperatures. Curve 5, vapor pressure of ether calculated from the theoretical lowering due to the water in the ether-water layer, which would hold for ether over water-ether layer since they are in equilibrium.

The crosses represent the partial pressure of ether at saturation calculated from the distribution ratios determined on dilute solutions.

separation out of the water (the so called "synthetic method" of determining solubility). From the solubility tables of water in ether the percentage water in the solution was estimated. The result showed that the partial pressure of ether as estimated was equal to that observed. This would be expected theoretically from the formula for the molecular lowering. The lowering of the vapor tension of water would be so small that it is safe to neglect it and to assume the vapor tension of water to be the same as over water at that temperature.

SUMMARY.

1. A modification of the Nicloux method for the determination of ether is presented consisting of absorption in H_2SO_4 , dilution of the acid to 40 per cent, and oxidation to acetic acid by the addition of bichromate, the excess of which is determined by iodometric titration. The accuracy of the method for the analysis of air, water, and blood is ± 2 or 3 per cent.

2. Although a small proportion of ether tends to be oxidized to CO_2 in strong H_2SO_4 bichromate solution, conditions may be controlled by proper regulation of strength of acid and control of temperature so that oxidation stops with a quantitative formation of acetic acid.

3. The distribution coefficients of ether for air-water and air-blood have been determined and agree with data obtained from partial pressure curves of ether in an ether-water mixture at saturation. These data form the necessary preliminaries to the determination of the anesthetic concentration of ether, which is taken up in the following paper.

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ETHER ANESTHESIA.

II. ANESTHETIC CONCENTRATION OF ETHER FOR DOGS.*

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A review of the determinations of the anesthetic tension of ether, reported by various investigators, shows large discrepancies in the results obtained. Data compiled from the literature are given in Table I, according to which the lowest concentration of ether vapor which has been found to produce or maintain anesthesia in man and other animals varies from about 2 to 10 volumes per cent. If ether vapor is absorbed, as first pointed out by Snow in 1848, by simple diffusion, the concentration of ether in blood must vary in direct proportion with its concentration in the lung air—according to these results, a fivefold variation. But Nicloux working on dogs found that the margin between the anesthetic concentration in the blood and that causing respiratory failure was small; 105 to 110 mg. of ether per 100 cc. being the anesthetic value and 160 to 170 mg. causing respiratory failure.

If we calculate the concentration of ether in air which would be in diffusion equilibrium with the Nicloux values at 37.5°C., using a distribution ratio of 15 (1, 2), we obtain the following.

Anesthetic concentration:

0.110 per cent in blood \approx 2.52 vols. per cent in air, or 19.2 mm. tension.

Fatal concentration:

0.170 per cent in blood \approx 3.9 vols. per cent in air, or 29.7 mm. tension.

In marked contrast with these values are the results, for example, of Boothby (3), who finds that about 6.7 volumes per cent or 51 mm. tension is the optimum concentration for surgical anesthesia of human subjects with the Connell apparatus.

* Conducted with the aid of a grant from Mr. Edward Mallinckrodt, Jr.

TABLE I.

Investigator.	Date.	Anesthetic concentration.	Amount of ether in alveolar air.	Amount of ether in blood.	Animal used.	Depth of anesthesia.	Method of collection.	Method of analysis.
Snow (4).	1848	3.6*	9.4	136	Rabbit.			Not analyzed. Known amount of ether vaporized in bell jar.
Lassaigne (5). Bert (6).	1847 1881	3.19-3.62* 10.65* 6.25*	8.4-9.5 27.9 16.2	122-137 405 233	Dog. " and man. Mouse. Sparrow. Rabbit and cat. Man.	Light.	Air in spirometer. " " " "	
Spencer (7).	1894	3.72* 5.48*	9.8 14.4	142 209			Air in spirometer " "	Calculated. Combustion.
Dreser (8).	1896	3.10-3.62* 1.2-4.7*	8.1-9.5 3.3-13.0	117-138 49.5-195		Surgical.	From mask. Anesthetic apparatus. " "	Absorption with alcohol and water. Calculated. " "
Hennicke (9).	1895	3.2*	84	122	Rat.			
Kionka (10).	1899	2.1-7.9*	5.6-20.7	80.1-300	Rabbit.			
Honigman (11). Nieloux (12).	1899 1908	6.8-9.7*	17.6-25.0 9.4-10.2	255-362 105-110*	" Dog.	Complete narcosis.	Amount in blood.	Bichromate oxidation.

Madelung (13).	1910	3.6-6.3*	9.4-16.5	136-140	Rabbit.	No corneal reflex.	Mixture from spirometer.	Combustion.
Ritschel and Stange (14).	1913	10.2*	26.8	389	"	Corneal reflex just visible.	Mixture from apparatus.	Absorption with alcohol and water.
Boothby (3).	1913	6.7*	17.5	256	Man.	Surgical.	Connell apparatus.	Gas balance.
van Leeuwen (15).	1916		6.9	100*	Cat.	Loss of homolateral flexion reflex.	Amount in blood determined.	Bichromate oxidation.

* The data marked are taken from the original figures. The other data are calculated from these, corrections being made for change in volume in passing from room temperature to body temperature. The amount in blood was calculated from the distribution coefficient of ether at body temperature.

Assuming absorption to be by simple diffusion and accepting Nicloux's value as to the fatal blood concentration, it is evident that dogs would die from breathing, without dilution, the concentration prescribed by Boothby. The fact that the anesthetization of human patients with such ether-air mixtures is a relatively safe procedure, shows that there are large errors in the data or in the reasoning, and the matter obviously requires further investigation.

The discrepancies in the results of different workers for the anesthetic concentration of ether may be due to a number of causes: (1) Errors in the methods of collecting and sampling the air for analysis; (2) unreliable methods for determination of ether; and (3) variations in the depth of anesthesia.

Spencer (7) filled his spirometer by aspirating into it a known quantity of ether. From the volume of the spirometer he calculated the percentage composition of the mixture. Later he analyzed a sample of the gas by passing a known volume over a glowing coil of copper oxide. The air, after passing over calcium chloride to remove the moisture, was passed through caustic alkali where the CO_2 was absorbed and then through sulfuric acid to insure complete drying. From the amount of CO_2 formed during combustion he calculated the amount of ether oxidized. The amount of ether found by analysis was much smaller than that calculated. The discrepancy he considered due to absorption of ether by the water on the sides of the spirometer.

Dreser (8) analyzed in a Hempel apparatus the gas collected from a gas mask, assuming this to be the same as alveolar air. Alcohol was used in the absorption bulb and water as a leveling liquid, the water removing the alcohol vapor. However, the water would also absorb some of the ether before the gas could be measured, furnishing another source of error. Kochmann (16) modified this method using a Bunta burette and mercury for leveling medium. After removing the ether by washing the burette several times with alcohol and washing out the alcohol with water, the volume of the measured gas was taken over the water used for the final washing, correction being made for the height of the water column. Kochmann (16) also tried to absorb ether with paraffin. The absorption, however, was so slow that unless the ether containing air was shaken with the oil,

it was impossible to remove all the ether. The air bubbles remaining in the oil made accurate readings impossible.

Waller and coworkers (17) estimated the per cent of ether by means of a gas balance. Boothby (3) made use of a gas balance for determining the percentage of ether as delivered by the Connell (18) apparatus and used the results obtained from the calibrated apparatus in his estimations. Boothby assumes the air delivered by the apparatus to be the same as the alveolar air of the subject. Since there is no valve used at the outlet for exhaled air it seems probable that some air not containing ether would be drawn in through the unprotected opening for expired air. This objection would apply to masks as well as to the tracheal tube.

Nieloux (12) and van Leeuwen (15) analyzed blood for ether, both using the method of Nieloux. Their results are in substantial agreement. None of these investigators with the exception of van Leeuwen made any attempt to measure the depth of anesthesia.

In this paper we present data, obtained on dogs, as to the amounts of ether in blood and in air breathed during various degrees of anesthesia.

The problem of furnishing a mixture of ether and air of the same concentration over long periods of time, and at the same time a method by which this concentration might be varied at will, presented some difficulties. The Connell (18) apparatus is an ingenious device for this purpose and as calibrated by Boothby (3) seems to be accurate. One of these was not available and it was thought doubtful if it could be relied upon for the accuracy desired.

When air is bubbled through liquid ether, it becomes saturated with ether vapor and contains a definite concentration depending upon the temperature of the liquid ether. Due to the heat required for vaporization the temperature is so rapidly reduced that it is impossible to keep the ether at a constant temperature even in a thermostat. If air saturated with ether at a temperature slightly above 0° is passed through a series of flasks kept at 0° enough of the ether will be condensed to leave an atmosphere in which the partial pressure of ether is that existing at 0° . As a small amount of ether is being condensed in the first flask there

is a tendency to raise the temperature. However, since only a relatively small amount will be condensed the effect on the temperature will be slight and the last flask of the series can be kept at a temperature of 0°C . by the use of an ice and salt bath for hours with little trouble and insignificant fluctuations.

The air with a constant partial pressure of ether is admitted into a mixing chamber where it is diluted to the desired concen-

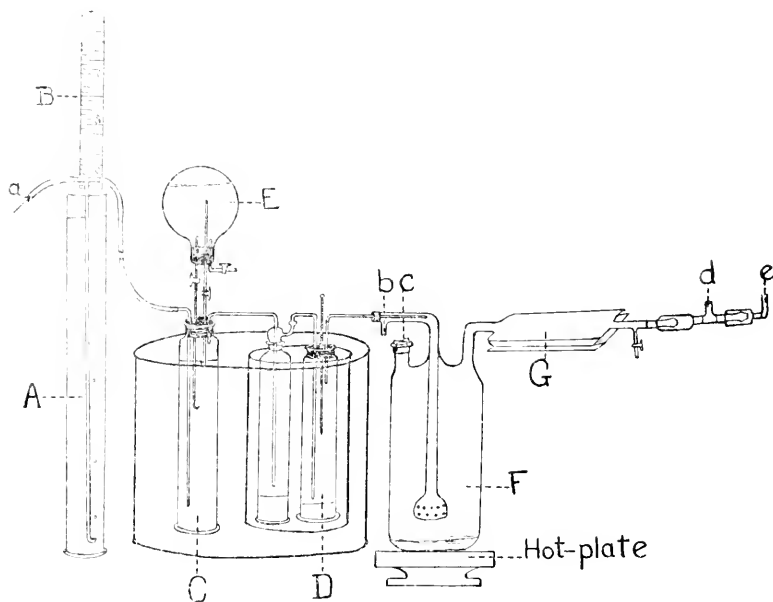


FIG. 1.

tration. This chamber is large enough to equalize any slight fluctuations arising from changes in air pressure. The chamber is kept in a water bath on a hot-plate which raises the temperature of the mixture to about room temperature between 22 and 25°C . A small amount of water in the bottom of the mixing chamber keeps the air saturated with water vapor. This water soon becomes saturated with ether so there is no observable effect on the ether tension.

Fig. 1 shows the apparatus used for this purpose. To regulate the rate of flow through the ether systems, air under pressure

is admitted at *a*. A water trap, *A*, is placed at the entrance of the system and the height of the column of water above the outlet regulates the flow of air through the ether. This is changed by raising or lowering the tube. The air pressure is always kept great enough to keep air bubbling slowly through the trap, fluctuations on the air line being thus compensated for. Variations are between 8 to 10 mm. of water and the size of the mixing chamber equalizes these. The height of the water column controlling the amount of air passing through the ether is calibrated in centimeters as measured on scale *B*. The air under pressure controlled by the trap passes through liquid ether in *C* where it becomes saturated. The ether in flask *C* is kept at a constant height from a supply flask, *E*, which is connected by means of two glass tubes, one extending to the bottom of the flask *C*, the other to the desired level of the ether and to the bottom of the inverted flask *E*. The ends of these tubes extending into flask *C* are protected from bubbles of air by being drawn out to capillaries and turned up. When the ether falls below the level of the tube air enters the flask *E* forcing ether into flask *C*. This automatic control works well if flask *E* is kept at a constant temperature. If it becomes heated the ether expands and is forced into flask *C*. This flask is in a water bath by which the temperature may be kept slightly above 0°C. The air saturated with ether then passes through two absorption flasks kept in an ice-salt bath. In the second of these is a thermometer which is kept at 0° by adjusting the ice-salt mixture. Both of these flasks are tall and slender, the inlet tubes are far enough from the bottom to allow the ether which condenses to accumulate without interfering with the pressure in the system.

Air saturated with ether at 0° leaves flask *D* and passes into mixing chamber *F* where it is mixed with air. The current of air passing into this chamber at *b* is kept constant by a device similar to that used for the ether line (not shown). Desired changes in concentration of the ether are made by varying the rate of flow through the ether system. There is a safety valve, *c*, on the mixing chamber. The air passes into a bag of parchment, *G*, which serves as a tension equalizer. A manometer placed beyond the tension equalizer shows variations of less than 10 mm. of water, which come within the limits of the de-

termination of ether. The animal used is connected to this apparatus by means of a tracheal cannula, *d*. Two Tissot valves protect the outlet and inlet, the dead air space is reduced to a minimum, and the expired air is carried to the outside air by means of a tube, *e*. Between the tension equalizer and the valve is an outlet through which samples of air are taken for analysis. The apparatus is calibrated empirically, so that the desired concentration may be had at any time. Check determinations have also been made on a sample of the air taken before and after blood determinations. Flow meters as described by Marshall and Kolls (19) may be used in each circuit in addition to the water trap to observe the maximum variations in pressure.

In Series I the dogs were anesthetized and after ligating the carotids, decerebrated by trephining and spooning out the two hemispheres. Pressure on the vertebrae until after a clot had been formed succeeded in stopping bleeding in all but one case. In two cases though the bleeding stopped the blood pressure fell to a very low level. The animals showed no rigidity though they lived for several hours. The results on these animals were discarded as obviously the operation had resulted in shock.

Reflexes were studied by the Sherrington method (20). The homolateral flexion reflex was registered. A Sherrington (15, 21) glass electrode was used for stimulation which was by means of induction shocks. Both make and break shocks were used and the coil of a Harvard inductorium was arranged to give a maximum contraction at break.

The temperature of the animals was kept constant by means of an electric heating pad controlled by a bimetallic thermostat placed in the rectum of the animal. The temperature could easily be controlled within 0.5°C . This temperature control is essential in working with decerebrate animals, though it is also important for the normal animal since anesthesia is always accompanied by a fall in temperature.¹

The concentration of ether was increased until the homolateral flexion reflex disappeared. With each change in concentration of ether the animal was allowed to breathe the same mixture for from 20 to 30 minutes before samples were taken, except

¹ This device for controlling temperature was worked out by Dr. G. H. Bishop.

where the reflex disappeared sooner. The concentration of ether in the blood and in the air breathed was determined at the same time. Duplicate samples of air were taken before and after the blood was drawn.

The second series was conducted in a similar fashion, except that the animals were not decerebrated and could not be allowed to come completely out of narcosis.

Determinations of ether in air and in blood were made by the modification of the Nieloux method reported in the preceding paper (1).

For collecting the samples of blood a needle was attached by a short rubber tube to a pipette calibrated in cubic centimeters, but not for complete delivery so that the last portion of blood could always be discarded. The needle was inserted into the artery or vein and the blood allowed to flow into the pipette. In case of venous blood a small amount of suction was usually necessary. The blood flow in the artery or vein was stopped for a short time after removing the needle; this allowed a clot to form, closing the hole made by the needle, and the blood was allowed to resume its flow. The blood was measured into flasks already prepared, in such amounts that would give between 2 and 3 mg. of ether for a determination. 2 or 3 cc. during complete anesthesia were sufficient. In experiments on elimination of ether larger samples were required as the ether became less concentrated in the blood. Ether is reported as mg. per 100 cc.

The air was collected over mercury at room temperature and forced slowly through H_2SO_4 , two tubes being used to insure complete absorption. The concentration is calculated to the basis of alveolar air; that is, saturated with water at the temperature of the animals. Protocols of the individual experiments are included at the end of the paper.

Relation between the Alveolar Concentration and the Amount of Ether in the Blood.—It is apparent from these experiments that the concentration of the ether in arterial blood varies directly with the concentration of ether in the alveolar air and depends upon the distribution coefficient at that temperature. The ratios shown actually to exist in the body during anesthesia compare well with those reported (1, 2) *in vitro* at the same temperature. Commonly, the ratios are slightly higher than those observed *in vitro*,

and a suggestion as to the explanation of this difference was the finding of a marked lipemia in the case of Dog VII, which showed an exceptionally high and increasing ratio. A determination by the Bloor method gave 0.94 per cent fat in this experiment, as compared with 0.54 per cent in the blood of Dog XI, with a "normal" distribution ratio. Data from these two experiments are given in Table II. It will be noted that the anesthetic concentration of ether in the alveolar air of Animal

TABLE II.
Effect of Lipemia on the Distribution Ratio.

Dog XI.				Dog VII.			
Ether in blood.	Ether in air.	Distribution ratio $\frac{\text{Amount in blood}}{\text{Amount in air}}$	Tem- pera- ture.	Ether in blood.	Ether in air.	Distribution ratio $\frac{\text{Amount in blood}}{\text{Amount in air}}$	Tem- pera- ture.
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		<i>°C.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		<i>°C.</i>
130	9.6	13.55	39.0	150	10.9	13.8	39.0
136	10.2	13.2		150	10.9	13.8	
138	10.2	13.5		160	11.7	13.7	
136	10.2	13.3		190	12.9	14.7	
137	10.2	13.4		193	12.7	15.2	
142	10.5	13.5		202	13.4	15.1	
142	10.5	13.5		220	14.3	15.4	
163	12.2	13.35		230	15.3	15.0	
156	11.4	13.7		165	11.0	15.0	
155	11.5	13.50		232	15.3	15.1	
156	11.5	13.55	39.0	236	15.3	15.4	39.0
155	11.5	13.50		237	15.3	15.5	
Fat on last sample of blood. 0.64				Fat on last sample of blood. 0.94			

VII compares well with that of other experiments, showing that although the concentration in the blood is high this does not affect the concentration in the tissues since the extra amount in the blood is held by fat which has a higher solubility for ether than the blood or the tissues. Table II shows this effect. If the change in the distribution ratio is due to fat the figures show an increase from 13.8 to 15.5 in the 5 hours of the experiment. This would indicate a progressive lipemia with the prolonged anesthesia. This was observed in only one animal.

In two experiments the effect of temperature is pronounced (Dogs I and III, Table III). The higher the temperature, the lower the distribution ratio. In Dog I the temperature gradually fell from 39.5 to 36.0 due to the fact that there was no attempt made to control it, and the distribution ratio rose accordingly. Dog III was apparently not completely decerebrated since he showed movements of front legs and shoulders until the anesthesia became deeper. In the first part of the experiment the temperature was 41°C. After movements stopped it fell to 39°C. and was held there for the remainder of the experiment by the aid of the thermoregulator.

TABLE III.
Effect of Temperature on Distribution Ratio in Vivo.

Dog III.				Dog I.			
Tem- pera- ture.	Ether in blood.	Ether in air.	Distribution ratio $\frac{\text{Amount in solution}}{\text{Amount in air}}$	Tem- pera- ture.	Ether in blood.	Ether in air.	Distribution ratio $\frac{\text{Amount in solution}}{\text{Amount in air}}$
°C.	mg. per 100 cc.	mg. per 100 cc.		°C.	mg. per 100 cc.	mg. per 100 cc.	
41	110	8.8	12.5	39.5	103.9	7.65	13.6
41	121	9.5	12.7	39.5	119.0	8.90	13.4
39	127	9.5	13.3	38.2	132.2	9.58	13.8
39	119	8.8	13.5	38.0	140.2	9.68	14.5
39	125	9.2	13.6	37.0	160.0	10.95	14.5
39	135	10.0	13.5	36.0	165.0	11.0	14.9
39	165	12.5	13.2	36.0	165.0	10.9	15.1
39	169	12.5	13.5	36.0	168.0	11.3	14.9

*Concentration of Ether Necessary to Cause a Disappearance
of the Reflexes.*

It was at first thought that the condition of the reflex would be a safe and reliable method for producing the same stage of narcosis in various animals. The disappearance of the corneal reflex to mechanical stimulation was used in the animals that were not decerebrated. The concentrations necessary to suppress this completely varied in three of the animals used from 110 to 130 mg. per 100 cc. of blood. In the fourth animal the reflex did not disappear until the concentration was 162 mg. per 100 cc. of blood (Dog V). Nicloux (12) and van Leeuwen (15) give

100 and 110 mg., respectively, as the concentration necessary for narcosis. This indicates that the response of the corneal reflex to mechanical stimulation is unreliable as an indication of the depth of narcosis.

The disappearance of the homolateral flexion reflex to electrical stimulation was used in all other experiments as described. After decerebration the strength of stimulus necessary to produce a maximum contraction was determined. The concentration at which a reflex to this stimulation could no longer be induced was determined and marked "no reflex" in the tables. This is taken as a measurement of the depth of anesthesia. In most cases if the strength of stimulus was increased at this point a reflex was again initiated. Though Sherrington electrodes were used in many cases, changing the position of the electrode on the nerve would allow the same strength of stimulus to produce a reflex. The complete block of a stimulus of any strength very closely approaches the point at which respiration is inhibited. The homolateral flexion is the simplest type of reflex, involving at most only a few neurones and synapses, and so it would be expected to disappear later than the more complicated reflexes.

The concentration at which stimulation of the saphenous nerve ceased to cause a reflex stimulation of the respiration was 139 and 142 mg. per 100 cc. of blood in the two animals tested. This points to a variation in the disappearance of different reflexes with different degrees of anesthesia. In these same animals the homolateral reflex disappeared at 150 and 151, respectively. In these two cases, at least, the reflex stimulation of the respiration due to stimulation of the central end of the saphenous nerve was suppressed before the homolateral flexion reflex.

In all these cases the response to reflexes was greatly reduced to ordinary stimulation long before the reflexes disappeared to strong stimulation. The difficulty of measuring the response quantitatively and of being sure that the condition of the nerve allows maximum stimulation makes this an inaccurate measurement of the depth of narcosis. Sensory stimulation of the skin might prove a more efficient method and less apt to cause degeneration of the nerve. There is also apparently a time element; a concentration which at first did not block the reflex after a time did so. For example in Experiment XI a concentration of

155 mg. did not block the reflex until after $1\frac{1}{2}$ hours. This final blocking was not due to local effect on the nerves since a new place was stimulated without response.

The concentration of ether at which respiration is paralyzed depends upon the duration of the anesthesia. No experiments were conducted which would give exact data on this point. In several animals anesthesia was induced by cone and drop method till respiration was stopped. Blood was taken from the femoral artery and the amount of ether determined. The animals usually

TABLE IV.

Concentration of Ether Necessary to Cause Respiratory Paralysis in Varying Lengths of Time.

Dog No.	Amount of ether in 100 cc.		Time under concentration given in Column I.		Total time of experiment.	
	Blood.	Air.				
	mg.	mg.	hr.	min.	hr.	min.
I	168-177*	11.4-13.2	3		8	
II	195	14.0	1		4	45
III	169	12.5	1		6	30
V	232	17.2		30	6	40
VII	237	15.3	1	20	6	50
VIII	179	13.0		15	3	45
IX	182	13.6		33	7	20
X	170	12.6		20	4	30
XI	155		4		10	10
XII	224			15		
XIII	202			22		
XIV	198			18		

* Animal died while the operator was absent. The concentration in air had risen from 11.4 to 13.2.

recovered when artificial respiration was resorted to or when given several blasts of CO₂. The data on these three cases, Dogs XII, XIII, and XIV, are found in Table IV and are higher than other concentrations reported, with the exception of Animals V and VII. The high concentration in No. VII is accounted for by a lipemia. However, the distribution coefficient for Animal V indicates that there was no lipemia, and the high figures in this case remain unexplained. These show considerable variation from the results of Nieloux and van Leeuwen. The lowest observed figure for respiratory failure is 155 mg.

per 100 cc. of blood (Dog XI), after an anesthesia of varying depths for a period of 10 hours. 4 hours of the time the concentration was stationary at 155 mg.

Relation between the Amount of Ether in Venous and Arterial Blood.

During the induction of anesthesia and while the anesthetic tension in the air supplied remains constant the venous blood always contains less than the arterial blood. This would indicate that the depot fat does not become saturated with ether during a period of several hours, but continually absorbs more. It may be that the increasing effect of a given concentration when long continued is due to an accumulation of ether in the lipoids of the cells. When ether is being eliminated the concentration in the venous blood is higher than that in the arterial blood. Nieloux's (12) observations that the fat around the kidney has a much higher concentration of ether than that under the skin is evidence that the fatty tissue becomes gradually saturated, depending largely on its relative blood supply.

The Elimination of Ether.—Since there is no evidence of destruction in the body, elimination must take place almost wholly through the lungs with the exception of the small amount excreted in the urine. The rapid loss through the lungs depends on the distribution coefficient for ether between air and blood. The fall in concentration in blood when the administration is discontinued is rapid. Herein lies the safety in the use of inhalation anesthetics; though the range between anesthetic and dangerous concentrations is narrow the drop to a safe concentration is rapid enough to prevent fatalities if the inhalation be stopped when the danger point is reached. That the rate of elimination depends on ventilation is shown by the curves (Fig. 2 and Table V). In Curve I respiration was stimulated by several blasts of CO_2 sent into the lungs by means of a bellows as the ether was removed. The animals whose elimination curves are shown in Nos. II and III received no CO_2 .

The time required for the complete elimination of ether depends not so much on the concentration in the blood as upon the duration of the anesthesia. The animal whose curve of

elimination is represented in No. I had been anesthetized for 3 hours and 15 minutes before elimination was started. Dog X, Curve II, was under only 40 minutes and Dog VIII, Curve III, had been under for 1 hour and 30 minutes. The curve shows

TABLE V.
Elimination of Ether.

Animals were decerebrated and anesthetized for varying lengths of time previous to experiment on rate of elimination. Dog X was anesthetized deeply for 40 minutes. No. IX had been under ether for 3 hours and 15 minutes. The respiration had been stopped once, but stimulated by several blasts of CO₂ from a bellows. No. VIII was in deep narcosis for 1 hour and 30 minutes.

Time after cessation of ether.	Per 100 cc. blood.					
	Dog X, Curve II.		Dog IX, Curve I.		Dog VIII, Curve III.	
	A*	V	A	V	A	V
<i>min.</i>	<i>mg.</i>	<i>mj.</i>	<i>mj.</i>	<i>mj.</i>	<i>mj.</i>	<i>mj.</i>
	140	136	135†	131	175	168
3	108	110				
4			66		120	140
6	94	86				
10	80	77	50†	54		
15	71	69				
20	63				79	88
21		46	30	36		
30	44					
40			20	25	47	53
60	24		17	22	31	34
90	15		12	18	18	
120	6		10‡			
150			10‡			

* A represents arterial; V, venous.

† CO₂ given by bellows

‡ Blood pressure low; rate of elimination may have been decreased due to circulatory failure.

that Dog X, under only 40 minutes, eliminated practically all the ether in 2 hours, while in Dog IX the blood still contains 10 mg. per 100 cc. at the end of 2½ hours, though it dropped much more rapidly to begin with due to respiratory stimulation. These curves of elimination compare well with those of Nieloux (12).

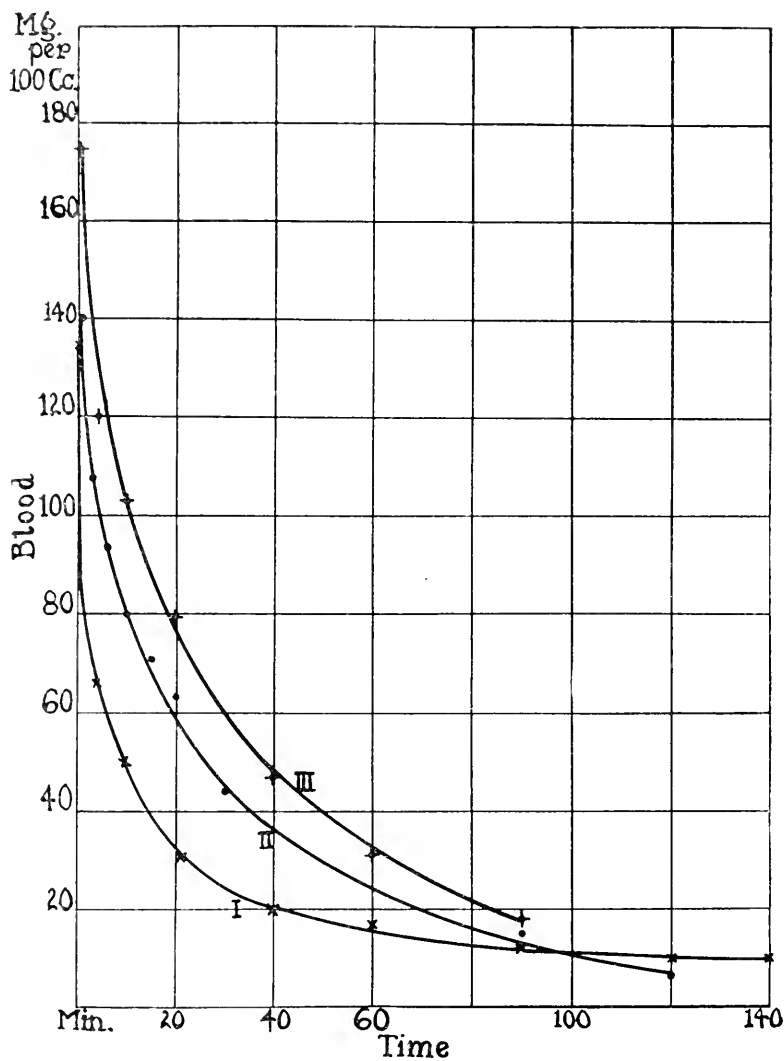


FIG. 2. Elimination of ether.

Protocols.

Experiment 1.—Dog I. Male. 4 kilos. 10.30 a.m. anesthetized. 11.00 a.m. Decerebrated, good rigidity. Excellent condition, little bleeding. 11.30 a.m. Attached to apparatus.

Time.	Re- spira- tory rate.	Blood pres- sure.	Pulse rate.	Tem- pera- ture.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>a.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mm.</i>	<i>mm.</i>	
11.30	36	170	114	39.5	50		
<i>p.m.</i>							
12.00				39.5	103.2	7.65	
12.30	36	170	114	39.5	103.9	7.70	
1.00				39.5	119.0	8.90	Slight reflex.
1.30	36	170	114	39.5	119.0	9.00	" "
2.00				38.2	132.2	9.58	
2.30	32	170	114	38.0	140.2	9.68	Slight reflex.
3.20	30	170	114	37.0	160.0	10.95	No reflex.
4.00	28	150	124	36.0	165.0	11.0	" "
4.20	26	150	120	36.0	165.0	10.9	" "
5.00	24	150	130	36.0	168.0	11.3	" "
5.50	24	140	130	36.0	168.0	11.4	" "
7.00						13.2	Animal left with ether going. The temperature had risen in system and concentration of ether furnished the dog had increased. This may have accounted for the death, or the fall in the temperature may have been a factor.

Experiment 2.—Dog II. Female. 6 kilos. 10.30 a.m. Anesthetized. Decerebrated. Bled but little. Decerebrate rigidity did not appear. Blood pressure low. Temperature 39°.

Time.	Re- spira- tory rate.	Blood pres- sure.	Pulse rate.	Tem- pera- ture.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>p.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mg.</i>	<i>mg.</i>	
12.00	50	110	96	39	163.5	12.2	
12.40	50	120	120		154.0	11.2	
1.00	50	140	120		169.5	12.5	Slight reflex.
1.40	30	110	120		189.0	14.0	No reflex homolateral.
2.20	20	108	140		190.0	14.0	" " "
2.40	20	110	90		195	14.0	" " "
3.00	10	100	90		195	14.0	
3.15				39	195	14.0	Respiration stopped. Heart continued to beat for about 5 min. after respiration stopped. Tried to bring animal around by giving CO ₂ by means of a bellows but was not successful. Blood pressure had dropped to about 40 mm. before this was tried.

Experiment 3.—Dog III. Male. 6.6 kilos. 10.00 a.m. Anesthetized. 10.30 a.m. Decerebrated. Apparently not complete; showed movements of front limbs; and rigidity did not appear. Animal attached to the apparatus.

Time.	Re- spira- tory rate.	Blood pres- sure.	Pulse rate.	Tem- pera- ture.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>p.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mj.</i>	<i>mj.</i>	
12.30	66	190	246	41	110	8.8	High temperature due to movements.
12.40				41	110	8.8	Movements ceased. Reflex to electrical stimulation good. Changed at 12.40 p.m.
1.10	60	170	240	41	120	9.5	Slight reflex.
1.30				41	121	9.5	“ “ Changed at 1.40 p.m.
2.00	60	120	242	39	127	9.5	
2.20					127	9.5	Reflex gone. Changed at 2.30 p.m.
3.00	60	110	242	39	119	8.8	Reflex returned. Changed at 3.10 p.m.
3.40	60	95	220	39	125	9.2	Reflex slight. Changed at 3.50 p.m.
4.00	60	90	228	39	135	10.0	Reflex gone.
4.30	78	70	246	39	165	12.5	
4.50	22	46	220	39	169	12.5	
5.10	15	40	120	39	169	12.5	
5.30		30	100 stopped.	39		12.5	

Experiment 4.—Dog IV. Male. 4.1 kilos. 8.35 a.m. Anesthetized. 9.10 a.m. Decerebrated; bleeding mild, easily stopped; animal in good condition; showed rigidity. 10.30 a.m. Ether started.

Time.	Respira- tory rate.	Blood pres- sure.	Pulse rate.	Tem- pera- ture.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>a.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mm.</i>	<i>mm.</i>	
10.00	42	128	180	39			Coil set so as just to give a reflex at make and maximal contraction at break.
11.30	42	128	180		111.8	8.4	Good reflex.
<i>p.m.</i> 12.00	20	138 120-140	120		134.6	10.5	Hemorrhage from brain increased intracranial pressure; blood pressure high; pulse rate irregular; Cheyne-Stokes respiration; pressure relieved; blood pressure returned to a little below normal; good reflex at make and break.
1.30	60	90 90	190 190	39	136.0 137.0	10.5 10.5	
2.30	60	100	182		153.6 153.2	11.5 11.5	Good reflexes. Make and break.
3.30	42	80	200	39	156.5 157.0	11.8 11.8	Reflex all right; diminished. Blood pressure falling.
3.40	20	60	90				Vagus stimulation; heart slow; reflex still present though diminished; death not due to anesthetic. Autopsy showed hemorrhage into brain cavity, completely filling it, and probably causing increased pressure on the medulla.
3.50	12	60	78				
4.00 stopped.		40	70	39	158.8	11.8	

Experiment 5.—Dog V. Female. 6.2 kilos. 9.00 a.m. Anesthetized. Tracheal cannula. Carotid blood pressure. Respiration recorded.

Time.	Respiratory rate.	Blood pressure.	Pulse rate.	Temperature.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>a.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mm.</i>	<i>mm.</i>	
9.30	96	166	208	39.5	162	12.2	Corneal reflex disappeared.
9.40	96	150	246		162	12.2	
10.00	92	134	240		150	11.35	Attached to apparatus; corneal reflex.
10.50					157	11.66	No homolateral reflex.
11.20	90	134	238		171	12.9	No corneal reflex.
11.40					182	13.5	" " "
<i>p.m.</i>							
12.20	90	134	240		222	16.5	No homolateral reflex.
12.40					223	16.5	
1.00	98	138	240		200	14.8	No homolateral reflex.
1.40					176	13.0	" " "
2.00							Decerebrated; when too far under, never recovered completely; showed no decerebrate rigidity; no reflexes.
2.30	24	80	252		237	17.6	
3.00	24	80	252		238	17.8	
3.30	50	70	252		232	17.2	
3.40	20	30	200	39.5			Dead.

Experiment 6.—Dog VI. Male. 8.2 kilos. 12.30 p.m. Decerebrated. Used for gum acasia experiment. 2.00 p.m. Attached to apparatus. Reflex good, decerebrate rigidity.

Time.	Respiratory rate.	Blood pressure.	Pulse rate.	Temperature.	Ether per 100 cc.		Remarks.
					Blood.	Air.	
<i>p.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mm.</i>	<i>mm.</i>	
2.00	30	120	156	39.5	108	8.2	Reflex good.
2.30	24	100	162		116	8.7	
3.00	28	100	160		123	9.1	
3.30	28	100	160		135	10.1	Homolateral reflex disappeared.
4.05	20	76	200		146	10.8	
4.35	20	70	210		162	12.0	
5.00	15	40	200	39.0	179	13.3	Respiration stopped.

Experiment 7.—Dog VII. Male. 8.1 kilos. 9.00 a.m. Anesthetized. Tracheal cannula. Blood pressure carotid. Homolateral flexion reflex registered. Respiratory rate; pneumograph. Blood examined for fat by Bloor's method.

Time.	Pulse rate.	Blood pressure.	Respiratory rate.	Temperature.	Ether per 100 cc.		Distribution ratio.	Remarks.
					Blood.	Air.		
<i>a.m.</i>	<i>per min.</i>	<i>mm. Hg</i>	<i>per min.</i>	<i>°C.</i>	<i>mg.</i>	<i>mg.</i>		
9.30	140	120	38	39	150	10.9	13.8	No corneal reflex.
10.00	140	120	40		150	10.9	13.8	" " "
10.20	140	120	40		160	11.7	13.7	" " "
11.00	160	150	36		190	12.9	14.7	Homolateral reflex present if stimulation increased.
11.40	140	150	30		193	12.7	15.2	
<i>p.m.</i>								
12.20	140	150	30		202	13.4	15.1	
12.50	140	150	30		220	14.3	15.4	
1.20	180	150	30	39	230	15.3	15.0	No reflex.
2.00	150	160	40		165	11.1	15.0	Reflex appeared.
2.30	120	120	25		232	15.3	15.1	No reflex.
3.00	100	110	20		236	15.3	15.4	
3.40	90	100	18		237	15.3	15.5	
3.50	90	40		39		15.3		Respiration stopped.

Experiment 8.—Dog VIII. Male. 6.3 kilos. 9.30 a.m. Decerebrated. Good rigidity. Venous blood from femoral vein. Arterial blood from femoral artery. Arterial drawn first.

Time.	Temperature.	Ether per 100 cc.			Remarks.
		Blood.		Air.	
		Venous.	Arterial.		
<i>a.m.</i>	$^{\circ}\text{C}.$	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.10	39.0	162	169	12.5	No reflex to mechanical stimulation.
10.30		165	169	12.5	No reflex.
10.55		158	150	11.2	" "
11.20		147	150	11.2	" "
11.40		168	175	13.0	" " respiration slow; ether stopped.
11.44		140	120		Reflex.
<i>p.m.</i>					
12.00		88	79		
12.40		53	47		
1.00		34	31		
1.30			18		
1.35					Ether started. Respiration very slow.
2.00		168	179	13.0	
2.15	39.0	172	179	13.0	Respiration stopped.

Experiment 9.—Dog IX. Male. 8.2 kilos. 9.30 a.m. Decerebrated. Good rigidity. Venous and arterial blood from femorals. Arterial drawn first.

Time.	Tem- pera- ture.	Ether per 100 cc.			Remarks.
		Blood.		Air.	
		Venous.	Arte- rial.		
<i>a.m.</i>	°C.	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.00	39	100	108	8.0	
10.30		112	120	8.9	
11.05		138	142	10.5	No reflex respiratory stimulation when saphena tetanized.
11.25		186	190	14.0	Respiration stopped. CO ₂ administered.
11.35		115	110		Blood taken. Receiving no ether.
<i>p.m.</i>					
12.30		128	131	9.7	
12.50		148	150	11.1	No homolateral flexion reflex.
1.15		131	135	10.2	Ether removed. CO ₂ administered.
1.19			66		
1.25		54	50		CO ₂ administered.
1.46		36	30		
1.55		25	20		
2.15		22	17		
2.45		18	12		
3.15			10		
3.45			10		
3.50					Ether started.
4.23		171	182	13.6	Respiration stopped 33 min. after starting.
4.40	39	179	182		

Experiment 10.—Dog X. Male, 8 kilos. 9.30 a.m. Decerebrated. Good rigidity. Reflexes not recorded. Arterial drawn first. Femoral venous followed by internal jugular venous. As much time as 1 minute may have elapsed between collection of each sample.

Time.	Tem- pera- ture.	Ether per 100 cc.			Remarks.
		Blood.		Air.	
		Venous.	Arte- rial.		
<i>a.m.</i>	<i>°C.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.00	39.5	107	110	8.2	
10.20		128	133	9.8	No reflexes.
10.40		128	136	10.4	
10.50		136	140	10.4	Ether removed. No reflex to mechan- ical stimulation.
10.53		110	108		
10.56			94		Reflexes appeared.
11.00		86	80		
11.05		77	71		
11.10		69	63		
11.20		46	44		
11.50			24		
<i>p.m.</i>					
12.20			15		
12.50			6		Determination questionable. due to small amount of ether. Ether started.
1.20			113	8.3	Reflexes to mechanical stimulation gone.
1.40			140	10.3	
2.00	39.5		170	12.6	
			170	12.6	Respiration stopped.

Experiment 11.—Dog XI. Male. 7 kilos. 9.20 a.m. Anesthetized. Tracheal cannula. No blood pressure.

Ether per 100 cc.					Tem- pera- ture.	Remarks.
Time.	Blood.			Air.		
	Fem- oral vein.	Inter- nal jugular vein.	Carotid artery.			
<i>a.m.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	°C.	
9.45	127	125	130	9.6	39	No corneal reflex.
10.00						Attached to apparatus.
10.30	130	130	136	10.2		No corneal reflex.
11.00	134	132	136	10.2		" " "
11.30	133	132	138	10.2		" " "
<i>p.m.</i>						
12.00	135	133	136	10.2		" " "
12.30	136	132	136	10.2		" " "
1.00	134	134	137	10.2		" " "
						Sciatic exposed. Preparations for registering homolateral reflex. Animal showed perfect relaxation during operation.
1.40	135	133	137	10.2		Homolateral reflex present.
2.20	138	137	142	10.5		" " "
2.50	140	139	142	10.5		Ether changed. No reflex stim- ulation to respiration.
3.30	160	158	163	12.2		No homolateral reflex.
3.50	156	154	155	11.4		Slight reflex.
4.00	154	153	155	11.4		" "
5.00	154	154	156	11.4		" "
5.30	153	154	155	11.5		No reflex.
6.00	155	154	156	11.5	39	" "
7.00	155	154	155	11.5		" " respiration very slow.
7.30	155	154	155	11.5		Respiration stopped.

SUMMARY.

1. A method is described for furnishing a constant tension of ether for an animal as large as a dog.

2. The concentration of ether in the blood is a function of that in the alveolar air and of the distribution coefficient at that temperature. The distribution coefficients determined in the body and *in vitro* are in substantial agreement. Certain abnor-

mal factors may influence the ratio; *e.g.*, an abnormally high percentage of fat.

3. Rate of elimination of ether depends on the respiratory volume and is greatly increased by respiratory stimulation due to CO_2 .

4. During the induction of anesthesia as already shown by Nicloux (12) the amount of ether is greater in the arterial blood and less in the venous. As long as ether of the same concentration is being administered the amount in the arterial blood remains higher than in the venous. This doubtless is due to the extreme solubility of ether in the depot fat.

During the elimination of ether the amount in the venous blood is greater. It is not all excreted in $1\frac{1}{2}$ hours due to the slow rate of removal from the fatty tissues. The length of time would depend on how completely these were saturated, upon their volume, and upon blood supply in relation to volume of respired air.

5. The disappearance of the homolateral flexion reflex in response to electrical stimulation of the nerve is variable, and disappears between 130 to 178 mg. per 100 cc. of blood. The corresponding concentrations in air breathed are (for $37.5^\circ\text{C}.$), 2.98 to 4.08 volumes per cent and 22.7 to 31 mm. of tension (Hg). Injury to the nerve and consequent variation in the strength of stimulus would account for some of the irregularities. Duration of the anesthetic is also a probable factor.

Corneal reflex to mechanical stimulation disappears between 110 to 130 mg. per 100 cc. of blood, which concentrations correspond (at $37.5^\circ\text{C}.$) to 2.52 to 2.98 volumes per cent and to 19 to 23 mm. of tension of ether vapor in the alveolar air.

6. Respiratory failure, if anesthesia is induced as rapidly as possible, may require as much as 190 to 237 mg. per 100 cc. (4.35 to 5.4 volumes per cent or 33 to 41 mm. of tension in alveolar air), but may be induced after several hours with concentrations from 155 to 195 mg. per 100 cc. of blood. (3.55 to 4.47 volumes per cent or 27 to 34 mm. of tension in alveolar air.)

7. The continued breathing by dogs of the concentration of ether in air stated by Boothby to be the anesthetic concentration for man (6.7 volumes per cent or 51 mm. of tension) produces a fatal concentration in blood. Assuming that the anesthetic

concentrations of ether in blood are the same for man as for dogs Boothby's estimate is much too high.

The reason that death does not result from the inhalation by human subjects of strong mixtures delivered by the Connell machine is doubtless that the air breathed is a mixture, in about equal parts, of that from the machine and of pure air. The prolonged inhalation of air from the machine (set for the above tension) without dilution would almost certainly prove fatal.

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CHEMICAL STUDY OF SEVERAL MARINE MOLLUSKS OF THE PACIFIC COAST.

THE LIVER.*

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The molluscan liver, also called hepatopancreas, functions not only as a center of production, storage, and conversion of complex carbohydrates but also serves the purpose of storing fat.

Voit (1) and Leydig in the report of their investigations on the pearl mussel drew attention to the importance of the liver as a fat-storing organ. Voit found that the liver of the above mentioned animal contained 9.7 per cent of fat, while the foot muscle contained only 4.3 per cent and the mantle 3.8 per cent. Levy (2) examined the liver of helix for fat, finding there only small quantities, while de Bellesme (3) found large amounts of fat in the liver of cephalopods. Deflandre (4) demonstrated the presence of fat in the liver of mytilus, but claimed that the amount varied with the season of the year. There is no doubt that the amount of fat in the liver decreases during starvation and increases on feeding fat-rich substances. Biedermann and Moritz (5) and Moritz (6) demonstrated the storage of fat in the liver, using snails as the experimental animals. They showed experimentally that when snails fasted until the liver was practically devoid of fat and were then fed on rich milk and cream, the liver was found to contain large amounts of fat a few hours after such feeding.

* This paper is a part of a thesis to the Department of Chemistry of Stanford University in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

TABLE I.

Constituents.	Abalone.	Pismo clam.	Cryptochiton.	Ischnochiton.
100 gm. of fresh liver tissue.				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Water.....	68.90	68.30	73.10	70.80
Total solids.....	31.90	31.70	26.90	29.20
Ash.....	5.79	5.93	3.38	9.12
Alcohol extractives, F1 + F2.....	15.10	15.50	8.24	12.16
Lipin fraction, F1.....	5.18	4.71	5.49	5.32
Water-soluble fraction, F2.....	9.92	10.89	2.75	6.84
Alcohol- and water-insoluble fraction, F3.....	16.01	16.16	18.10	17.76
Ash of F1 + F2.....	1.67	1.65	0.44	1.32
“ “ F3.....	4.12	4.28	2.94	7.80
Total N.....	1.86	3.43	0.57	2.35
Protein.....	11.85	21.85	22.74	14.97
Purine N.....	Trace.	Trace.	Trace.	Trace.
Creatine and creatinine.....	None.	None.	None.	None.
Uric acid.....	“	“	“	“
100 gm. of F1 + F2 + F3.				
Alcoholic extracts, F1 + F2.....	48.5	49.20	30.60	40.10
Alcohol-insoluble residue, F3.....	51.5	50.80	69.40	59.90
100 gm. of F1 + F2.				
Total solids, F1.....	34.3	30.20	66.60	42.20
“ “ F2.....	65.7	69.80	33.40	57.80
Ash of F1 + F2.....	16.5	16.30	16.70	19.30
“ “ F3.....	25.8	26.49	16.28	44.30
100 gm. of F1.				
Total N.....	1.44	0.66	0.84	0.57
Amino-acid N.....	0.157	0.069	0.0258	0.0932
Total sulfur.....	0.46	0.14	0.40	0.25
“ phosphorus.....	0.85	0.30	0.36	0.42
Lipin sugar.....	None.	None.	None.	None.
100 gm. of F2.				
Total N.....	6.70	2.26	14.0	6.34
Amino-acid N.....	3.60	0.90	4.20	1.32
Proteose N.....	0.21	0.20	1.20	0.70
Urea N.....		0.02	0.12	0.03

TABLE I—*Concluded.*

Constituents.	Abalone.	Pismo clam.	Crypto-chiton.	Ischno-chiton.
100 gm. of F2—Concluded.				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Urea		0.04	0.30	0.07
Ammonia	0.28	0.29	1.80	0.56
Total sulfur	3.64	1.19	9.40	8.14
Inorganic phosphorus	96.80	45.40	58.00	71.60
Creatine and creatinine	None.	None.	None.	None.
Uric acid	"	"	"	"
Reducing sugars after hydrolysis	29.00	15.40	28.60	16.80

100 gm. of F3.

Total N	12.63	5.74	6.08	7.24
Protein ($\times 6.37$)	72.11	35.95	38.93	45.25
Total phosphorus	0.63	0.71	2.90	1.62
Phospho-protein phosphorus	2.59	0.47	2.95	1.81
Total sulfur	2.28	1.10	2.31	1.62
Creatine and creatinine	None.	None.	None.	None.
Uric acid	"	"	"	"
Reducing sugars after hydrolysis	1.99	13.24	13.23	10.20

Inorganic constituents per 100 gm. of tissue.

SiO ₂	3.00	1.77	0.15	0.20
Fe ₂ O ₃	3.00	0.96	6.76	4.23
CaO	0.65	0.63	1.44	1.36
MgO	0.83	0.15	0.26	0.30

TABLE II.
Enzymes in Liver.

	Abalone.	Pismo clam.	Crypto-chiton.	Ischno-chiton.	Owl limpet.
Amylase	+	+	+	+	+
Catalase	+	+	+	+	+
Cytase	—	—	—	—	—
Emulsin	+	+	+	+	+
Glycogenase	+	+	+	+	+
Lactase	+	+	+	+	+
Lipase	+	+	+	+	+
Maltase	+	+	+	+	+
Protease	+	+	+	+	+
Sucrase	+	+	—	+	+
Urease	+	+	+	+	—
Uricase	+	+	—	—	—

Among inorganic substances iron seems to be of special importance in molluscan liver. Dastre and Floresco (7) interested in the so called "iron storing function of the liver" found that the liver of gastropods and lamellibranchiae contained four to six times as much iron as the rest of the body. They concluded that by far the largest amount of iron in the whole body is accumulated in the liver.

Bradley (8) reported the presence of metallic zinc in the liver of *Sycotypus canaliculatus*, from 11 to 23 per cent ZnO in the ash of the hepatopancreas.

The liver serves not only the purpose of storing carbohydrates, fats, and proteins, but secretes important digestive enzymes. Proteolytic enzymes have been reported in the liver juices of the oyster by Heyman (9) and Biedermann and Moritz. Krukenberg (10), Fredericq Heyman, and Griffith and Jung discovered the presence of carbohydrate-splitting enzymes in cephalopods and gastropods. Catalase was found in the liver of mytilus, limax, and helix by Zieger (11). Cellulase was not found in the liver juice by Biedermann and Moritz. The action of this enzyme was not noticed until it had been secreted, from which the conclusion might be drawn that an enzyme activator was necessary. Strong lipase action was observed in the liver extract of mollusks by Biedermann and Moritz. Of purine enzymes, Mendel and Wells found nuclease and amidase in sycotypus. Uricase was demonstrated in the liver extract of the shark by Scaffids.

The species of mollusks that were employed in this investigation are the abalone, Pismo clam, *Cryptochiton*, *Ischnochiton*, and owl limpet.

Methods of Analysis.

To determine the chief constituents of the liver, the same methods were employed as those used by the author (12) in the analysis of the muscle.

Owl limpet liver could only be examined for the presence or absence of enzymes. The specimens gathered did not yield sufficient liver tissue to carry on a more complete investigation.

DISCUSSION.

As compared with the composition of the muscle of these mollusks the analysis of the liver presents the following differences. The contents of water in the liver varies greatly from that in the muscle. The extractives with alcohol yield in abalone, Pismo clam, and cryptochiton approximately double the amount of extractives in the muscle, the larger amounts of fat present in the liver being the chief reason for it. The ash content in the liver is higher than in the muscle. Creatine and creatinine, which were not found in the muscle except in the abalone, are also absent in the liver. Cryptochiton liver leads with far the highest percentage in all the nitrogen compounds. The absence of urea in abalone liver is noteworthy.

If the urea had broken down into ammonia and carbon dioxide, we should expect a larger amount of ammonium salts, but the analysis does not bear this out. The amount of ammonia compared with that of the liver of vertebrates gives the following data.

	<i>mg.</i>
Abalone liver.....	28
Pismo clam liver.....	29
Cryptochiton liver.....	50
Ischnochiton liver.....	38
Vertebrate liver.....	22.8

Phosphorus, partly organic, partly inorganic, is built up into phosphonucleic acid and phosphatides, while the inorganic phosphates, as calcium phosphate, form the main part of the so called "Kalkzellen." Reducing sugars include pentoses and hexoses and are found in the tissue of mollusks. (Henze identified xylose in the octopus.) They are present in fairly large amounts in mollusks. Among the inorganic constituents we notice chiefly the large amount of iron.

The enzymes in the molluscan liver are practically the same as those present in the alimentary canal. This supports the suggestion that the enzymes are produced in the liver and secreted into the intestines.

SUMMARY.

In the liver of the mollusks studied, the high values found for ash in fresh tissue, ischnochiton 9.12 per cent, Pismo clam 5.93 per cent, abalone 5.79 per cent, and cryptochiton 3.38 per cent, are all the more interesting when the amount of iron present is considered. Calculated as ferric oxide, it makes up 31.9 per cent of the ash of the liver of the abalone, 31.7 per cent of that of the Pismo clam, 29.2 per cent of that of the ischnochiton, and 27.0 per cent of that of the cryptochiton. Uric acid, creatine, and creatinine were absent, but urea was found in all but the abalone. The liver is rich in enzymes, containing all those mentioned above as present in the alimentary canal, and, in addition, an active uricase in the abalone and the Pismo clam. These results lend emphatic support to the belief that the enzymes found in the stomach and intestines have their origin in the liver (or hepatopancreas) and not in the food ingested.

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CONCERNING THE ANTHOCYANS IN NORTON AND CONCORD GRAPES. A CONTRIBUTION TO THE CHEMISTRY OF GRAPE PIGMENTS.

By R. J. ANDERSON.

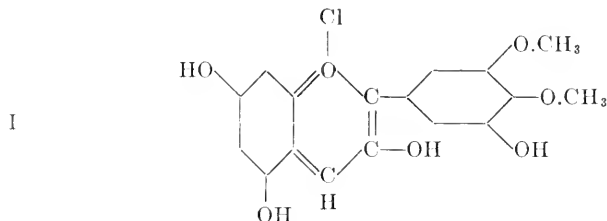
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INTRODUCTION.

It has been shown by Willstätter and Zollinger¹ that the pigment in the dark blue European grape, *Vitis vinifera*, consists largely of the monoglucoside oenin that they isolated in crystalline form as oenin chloride, $C_{23}H_{25}O_{12}Cl$. When hydrolyzed by boiling it with hydrochloric acid oenin yields 1 molecule of glucose and 1 molecule of the sugar-free pigment, oenidin chloride, $C_{17}H_{15}O_7Cl$. The above authors² have also shown that a diglucoside and some free oenidin is associated with the monoglucoside as it occurs in the grape.

Oenidin is regarded by the above authors as a dimethyl ether of delphinidin and they believe that its structure is represented by either Formula I or II.



¹ Willstätter, R., and Zollinger, E. H., *Ann. Chem.*, 1915, edviii, 83.

² Willstätter, R., and Zollinger, E. H., *Ann. Chem.*, 1916, edxiii, 195.

Color Reaction with Ferric Chloride.

Solvent.	Substance.	
	Anthocyanin.	Anthocyanidin.
Water.	The color changes to purplish, but fades quickly to light brown.	Momentary purplish color that fades immediately leaving a nearly colorless solution.
Ethyl alcohol.	Intense blue color changing in a few minutes to a rather permanent purple, but changing gradually in the course of several hours to wine-red.	Momentary purplish color that fades immediately leaving a faintly yellowish solution.

The anthocyanin chloride separated slowly from a mixture of alcohol and dilute hydrochloric acid in the form of dense cubical crystals. The substance was readily soluble in water giving a dull brownish red solution. In very dilute hydrochloric acid it dissolved with bright red color. It was very soluble in methyl alcohol, the solution exhibiting an intense and beautiful carmine-red color. It was readily soluble in ethyl alcohol giving a purplish red color.

The anthocyanidin chloride crystallized, on hydrolyzing the glucoside with boiling hydrochloric acid, from the hot solution in beautiful prism-shaped crystals. When the crystals were seen singly under the microscope the color was light brownish red. The macroscopic appearance of the dry substance was nearly black. It was easily soluble in methyl, ethyl, amyl, isopropyl, and butyl alcohol. The solutions exhibited beautiful shades of color from bright carmine-red in methyl alcohol to purplish red in amyl alcohol. It dissolved easily in water with dull brownish red color. In dilute hydrochloric acid it dissolved with dark red color, but in 3 per cent or stronger hydrochloric acid it was only slightly soluble.

By means of the "Distribution number" according to Willstätter and Zollinger² some evidence was found that the crude pigment in both the Norton and the Concord grapes contains not only the monoglucoside but also some diglucoside and some free anthocyanidin.

The spectrum of the anthocyanin forms one broad band extending from yellow into the blue. Unfortunately the band is too broad and the margins are too indefinite for the absorption spectrum to be utilized for the certain and rapid identification of the pigment.

EXPERIMENTAL PART.

Anthocyan in Norton Grapes.

Extraction of the Pigment with 1 Per Cent Hydrochloric Acid.

The skins only were used in the preparation of the pigment. They were separated from the pulp by hand and pressed in a small wine press. The juice contained only a very small amount of the coloring matter. The pressed grape skins, 2.2 kilos, were digested in 3 liters of 1 per cent hydrochloric acid over night. The skins were then rubbed into a pulp in a large mortar. The mixture was filtered on a large Buchner funnel and washed with 2 liters of 1 per cent hydrochloric acid.

The clear filtrate measured about 5 liters. It was of an intense and brilliant carmine-red color. After warming it to about 40° on the water bath it was mixed with 6 liters of a warm saturated aqueous solution of picric acid. After cooling to room temperature, the solution was allowed to stand in the ice box for 36 hours. The pierate separated slowly in the form of bright red needle-shaped crystals, usually arranged in fan-shaped or star-shaped aggregates.

The crystals were filtered off, washed free from picric acid with ether, and finally dried in a vacuum desiccator over sulfuric acid. The dry substance formed a dark red-colored mass and it weighed 11.4 gm.

The filtrate was very dark red in color. Concentrated hydrochloric acid was added to it until the solution contained 10 per cent of hydrochloric acid. On standing in the ice box over night a further quantity of dark red crystalline plates separated. This substance was filtered off, washed with a little cold 10 per cent hydrochloric acid, and dried in a vacuum over sulfuric acid and potassium hydroxide. The dry product was of purplish dark red color and it weighed 50.5 gm.

The filtrate was still very dark red in color, but no further crystals could be obtained and it was discarded.

The second crystalline product consisted evidently to a large extent of pieric acid. The free pieric acid was easily removed by digesting the crystals in warm benzene, filtering, and repeating the treatment. The benzene was finally removed by washing the residue with ether. The insoluble pierate was nearly black in color and after drying in a vacuum over sulfuric acid it weighed 5 gm. The total yield of the crude pierate amounted to 16.4 gm. or 0.74 per cent by weight of the pressed skins.

Isolation of the Glucoside by the Method of Willstätter and Zollinger.

The first pierate preparation, 11.4 gm., was reduced to a fine powder in a mortar and it was then dissolved in 250 cc. of methyl alcohol, forming a brilliant red solution. To this solution were added 50 cc. of methyl alcohol, containing 20 per cent of hydrochloric acid. The deep brilliant carmine-colored solution was filtered to remove a small amount of nearly colorless insoluble matter. The clear filtrate was mixed with 1,600 cc. of ether which precipitated the glucoside in the form of small dark red granular particles. After standing over night it was filtered and washed carefully with ether and dried in a vacuum desiccator over sulfuric acid and potassium hydroxide. The dry product formed a compact dark red mass that showed a beautiful metallic bronze luster in reflected light. It weighed 7.2 gm.

The ethereal solution, after filtering off the precipitated glucoside, contained a considerable amount of pigment that could not be precipitated by further addition of ether. The dissolved coloring matter was precipitated on the addition of petroleum ether as a dark red, thick, aqueous layer on the bottom of the container. This portion of the pigment was discarded.

From the second pierate preparation mentioned above, we obtained by the same method, 1.9 gm. of the glucoside. The total yield of the glucoside as chloride was, therefore, 9.1 gm. or 0.41 per cent of the pressed grape skins. This represents only a part of the pigment present in the original grape skins. The extraction of the pigment with dilute hydrochloric acid was not complete and a considerable amount was not precipitated by the

picric acid. A further loss was caused by the incomplete precipitation of the glucoside by the ether from the methyl alcohol.

When some of this crude pigment was dissolved in 0.5 per cent hydrochloric acid all the coloring matter could not be extracted by repeatedly shaking the solution with amyl alcohol, indicating the presence of some diglucoside. According to Willstätter and Zollinger² the diglucosides generally remain in the dilute acid. The purplish red-colored amyl alcohol obtained in the above extractions was repeatedly extracted with 0.5 per cent hydrochloric acid. A large part of the coloring matter, representing the monoglucoside, passed into the dilute acid, but it was impossible to remove all the pigment by this treatment. The coloring matter remaining in the amyl alcohol represents the free anthocyanidin.

Crystallization of the Anthocyanin Chloride.

We followed the procedure of Willstätter and Zollinger.¹ The crude glucoside, 3 gm., was digested in 10 cc. of methyl alcohol. It was not completely soluble and even after adding 25 cc. of ethyl alcohol and 5 cc. of 10 per cent hydrochloric acid and stirring for some time, a portion of the substance did not dissolve. The insoluble material was filtered off, washed in a few cubic centimeters of methyl alcohol, and dried. It weighed 0.8 gm. It formed a dark red granular powder. It was completely, but slowly, soluble in methyl alcohol when a sufficient quantity of this solvent was used and it was easily soluble in water. On combustion the substance burned completely, leaving no ash. After drying at 105° in a vacuum over phosphorus pentoxide it was analyzed.

Found. C 50.82, H 4.42 per cent.

Calculated for diglucoside, $C_{25}H_{35}O_{17}Cl$ (690.5). C 50.40, H 5.06 per cent.

Judging by its composition the substance appears to be a mixture of the mono- and diglucoside.

To the filtrate from the above insoluble matter, were added 5 cc. of 10 per cent hydrochloric acid. It was left in a loosely covered dish to crystallize at room temperature. The substance separated slowly, as the alcohol evaporated, in the form of cubical masses

of crystals. After it had stood for 3 days the substance was filtered off, washed with a little cold 10 per cent hydrochloric acid, and dried in a vacuum over sulfuric acid and potassium hydroxide. It weighed 0.6 gm.

The filtrate was allowed to stand for 2 days in a partially evacuated desiccator over sulfuric acid when a second crop of crystals separated. The crystal form was similar to that described above and consisted of superimposed cubical masses. The crystals were filtered off, washed, and dried as before.

The filtrate, which was very dark red in color, was saved for the preparation of the sugar-free pigment.

The dry anthocyanin chloride weighed 1.2 gm. It was analyzed after drying at 105° in a vacuum over phosphorus pentoxide. 0.1919 gm. of substance lost 0.0074 gm. or 3.85 per cent in weight which corresponds to 1 molecule of water of crystallization. Calculated for 1 H_2O , 3.31 per cent.

0.1845 gm. substance: 0.0767 gm. H_2O and 0.3527 gm. CO_2 . 0.1868 gm. substance: 0.0531 gm. AgCl .

0.2105 gm. substance: 0.0589 gm. AgCl .

Calculated for anthocyanin chloride, $\text{C}_{23}\text{H}_{25}\text{O}_{12}\text{Cl}$ (528.5). C 52.22, H 4.73, Cl 6.71 per cent.

Found. C 52.13, H 4.65, Cl 7.03, 6.92 per cent.

A second preparation of the glucoside was made by the method just described. From 5.1 gm. of the crude glucoside we obtained 3 gm. of the air-dried substance.

For analysis the substance was dried as mentioned above. The loss in weight was 8.73 and 8.74 per cent which corresponds to 3 molecules of water of crystallization. It is evident that the first glucoside preparation had lost 2 molecules of water of crystallization on drying in a vacuum over sulfuric acid at room temperature. Calculated for 3 H_2O , 9.27 per cent.

Analysis of the dried substance gave the following result.

Found. C 52.33, H 4.55, Cl 6.81 per cent.

Hydrolysis of the Glucoside.

Preparation of the Sugar-Free Pigment, Anthocyanidin Chloride.

The filtrate from the above anthocyanin chloride was diluted with 30 cc. of 25 per cent hydrochloric acid and the solution was

boiled over a free flame for 4 minutes. The anthocyanidin chloride began to crystallize, after the solution had been boiled for about 2 minutes, in prisms usually arranged in fan-shaped aggregates. Examined under the microscope the crystals appeared to be perfectly homogeneous; the color of single crystals was faint brownish red. After the solution had cooled the crystals were filtered, washed in a little cold 20 per cent hydrochloric acid, and dried. The dry substance appeared nearly black in color and it weighed 0.25 gm.

In another experiment 1 gm. of the glucoside was dissolved in 25 cc. of water, 25 cc. of concentrated hydrochloric acid were added, and the solution was boiled for 4 minutes. The anthocyanidin chloride crystallized, after boiling for about 2 minutes, from the hot solution in beautiful brownish red prisms as mentioned above. The substance was filtered, washed in cold 20 per cent hydrochloric acid, and dried in a vacuum over sulfuric acid. The dry crystals weighed 0.55 gm. The filtrate, which was of dark red color, was extracted with 100 cc. of amyl alcohol. The pigment passed into the amyl alcohol, leaving the dilute acid solution practically colorless. The amyl alcohol was washed with water and evaporated on the water bath. The dark red amorphous residue, after drying in a vacuum over sulfuric acid weighed 0.07 gm. The total weight of the sugar-free pigment was 0.62 gm. The theoretical amount obtainable from 1 gm. of monoglucoside is 0.69 gm.

The substance was analyzed after drying in a high vacuum over phosphorus pentoxide at 105°C.

0.1551 gm. substance: 0.0555 gm. H_2O and 0.3143 gm. CO_2 . 0.1347 gm. substance: 0.0530 gm. $AgCl$.

Found. C 55.26, H 4.00, Cl 9.73 per cent.

A second preparation obtained by the same method gave the following results.

Found. C 56.19, H 4.08, Cl 9.48 per cent.

A third and fourth preparation, after recrystallizing from diluted hydrochloric acid as will be described below, gave the following results.

Found. C 56.17, H 4.18, Cl 9.90 per cent.

“ “ 56.08, “ 4.15, “ 9.25 “ “

Calculated for anthocyanidin chloride, $C_{17}H_{15}O_7Cl$ (366.5). C 55.66, H 4.09, Cl 9.68 per cent.

Average of above analyses. C 55.92, H 4.10, Cl 9.69 per cent.

Water of Crystallization in Anthocyanidin Chloride.

Willstätter and Zollinger found that oenidin chloride contained 1.5 molecules of water of crystallization. The preparations which we obtained and analyzed lost weight on drying at 105° in a high vacuum over phosphorus pentoxide, corresponding to 1.5 molecules of H_2O as shown below.

Loss in weight: 7.62, 7.43, 6.65, 6.75 per cent. Average 7.11 per cent.

Calculated for 1.5 H_2O . 6.86 per cent.

Properties of Anthocyanidin Chloride.

The dry crystals are very dark, nearly black in color, and show a metallic bronze luster in reflected light. They were easily soluble in water, giving a brownish red solution; the solution was clear, but the color was dull. On acidifying the aqueous solution with a few drops of hydrochloric acid the color changed to bright red. It was easily soluble in ethyl alcohol, forming a brilliant clear purplish red color. It was easily soluble in methyl alcohol, giving a beautiful bright carmine-red color. It is only slightly soluble in hot or cold 3 or 4 per cent hydrochloric acid. After heating to boiling in 3 per cent hydrochloric acid and filtering nothing crystallized out on cooling. The dissolved pigment separated slowly from the above solution on adding concentrated hydrochloric acid, but the dark red or nearly black precipitate was not crystalline but consisted of very fine granular particles.

While the anthocyanidin chloride always separated in beautiful prisms on hydrolyzing the glucoside with hydrochloric acid, it was found to be very difficult to recrystallize it. Dissolved in ethyl or methyl alcohol and mixed with 10 per cent hydrochloric acid, the pigment always separated slowly, on standing for a few hours, in the form of amorphous round granular particles, and from aqueous solutions mixed with hydrochloric acid it precipitated in the same form after the solution had stood for a few hours.

It could only be recrystallized by dissolving it in water and then carefully acidifying until the solution contained 3 per cent of hydrochloric acid. From this solution there separated in the course of several hours small prismatic crystals, mixed with some fine granular particles. After filtering, washing with 3 per cent hydrochloric acid, and drying, the crystals were freed from amorphous particles by digesting them for a short time in a little cold isopropyl alcohol. On now filtering and washing with a little cold isopropyl alcohol the crystals were obtained perfectly free from any amorphous pigment.

Behavior of Solutions of Anthocyanidin Chloride towards Amyl Alcohol.

Anthocyanidin chloride, dissolved in water and acidified with hydrochloric acid, could be completely extracted with amyl alcohol. The amyl alcoholic solution exhibited an intense purplish red color.

If a small amount of calcium oxide, sufficient to bind the hydrochloric acid, was added to the brownish red solution of anthocyanidin chloride in water, the color changed to a beautiful blue. The same change in color was observed on adding just sufficient dilute ammonium or sodium hydroxide. On extracting these blue-colored solutions with amyl alcohol the aqueous layer became decolorized, the pigment passing into the amyl alcohol giving a beautiful purplish red solution. When an excess of the base was present, however, the amyl alcohol remained perfectly colorless and the aqueous layer was blue in color. It was possible to add the base in such proportions that a part of the pigment was extracted by amyl alcohol, giving a purplish red solution while a part remained in the aqueous layer which retained its blue color.

Absorption Spectrum of Anthocyanin Chloride.

The spectrum of anthocyanin chloride consists of one broad band extending from yellow into the blue. The following determinations were made and the results are similar to those reported by Willstätter and Zollinger for oenin chloride.

In Ethyl Alcohol. 1 Molecule in 2,318 Liters.

Column	2 mm.	581..567....532
"	5 "	593..583....506
"	8 "	597..588....493
"	10 "	599..590....487

In Methyl Alcohol. 1 Molecule in 1,522 Liters.

Column	1 mm.	574..558....518
"	2 "	576..564....509
"	3 "	585..574....492
"	5 "	589..578....477

In Water. 1 Molecule in 1,601 Liters.

Column	5 mm.	538..528....483
"	10 "	558..548....477
"	15 "	569..553....474

In 1 Per Cent Hydrochloric Acid. 1 Molecule in 1,650 Liters.

Column	2.5 mm.	548..532....487
"	3 "	554..537....486
"	4 "	565..543....470
"	5 "	566..546....464

In $\frac{1}{4}$ Per Cent Sulfuric Acid. 1 Molecule in 1,601 Liters.

Column	2 mm.	541..527....494
"	3 "	557..539....478

In 66 Per Cent Acetic Acid. 1 Molecule in 2,893 Liters.

Column	2 mm.	567..560....505
"	3 "	573..564....503
"	4 "	575..566....492
"	5 "	578..570....486

Methoxyl Groups in Anthocyanidin Chloride.

The anthocyanidin chloride used in these determinations had been recrystallized as described above and dried to constant weight at 105° in a high vacuum over phosphorus pentoxide. The usual Zeisel method was used.

0.2533 gm. substance: 0.1760 gm. AgI. 0.2023 gm. substance: 0.1485 gm. AgI.

Calculated for 1 CH_3O . 8.45 per cent.

" " 2 CH_3O , 16.91 " "

Found. CH_3O 9.17, 9.69 per cent.

Willstätter and Zollinger¹ reported results that indicated that the oenidin chloride derived from *Vitis vinifera* contained two methoxyl groups. It is evident from the figures given above that the amount of methoxyl found in our preparation corresponds

more nearly to $1 \text{ CH}_3\text{O}$, resembling in this respect the anthocyanidin isolated by the above mentioned authors² from *Vitis riparia*.

The mixture of hydriodic acid and acetic anhydride containing the demethylated pigment was dark red in color, and after cooling, some of the pigment was deposited as amorphous granular particles of dark reddish color. The residues from the above two determinations were united, filtered, washed with 20 per cent hydrochloric acid, and dried. The substance weighed 0.4 gm. It was dissolved in 10 cc. of alcohol, a few drops of concentrated hydrochloric acid and an excess of precipitated silver chloride were added, and the mixture was shaken for several hours. The insoluble silver salts were filtered off and washed with a little alcohol. To the filtrate were added 10 cc. of 20 per cent hydrochloric acid and the alcohol was evaporated slowly by warming the solution on the water bath. The solution was then allowed to stand at room temperature for 36 hours when the pigment separated slowly in the form of dark reddish brown fine granular particles. The substance was filtered, washed with dilute hydrochloric acid, and dried in the air; weight 0.2 gm. It was readily soluble in alcohol giving a purplish red color. The color was very permanent. The alcoholic solution, diluted with water, was boiled for some time and repeatedly evaporated on the water bath without showing any apparent loss in color. The substance did not show any tendency to crystallize. On slowly concentrating the solution in a desiccator only amorphous granular particles were obtained.

The substance was analyzed after drying in a vacuum at 105° over phosphorus pentoxide.

Found. C 56.44, H 3.63, Cl 10.47 per cent.

The properties and composition of this material differ decidedly from those of delphinidin chloride that Willstätter and Zollinger obtained from oenidin chloride. Judging by our analytical results the anthocyanidin chloride, during demethylation with boiling hydriodic acid and acetic anhydride, must have suffered some loss of oxygen; possibly due to conjugation with elimination of 1 molecule of water.

*Cleavage of Anthocyanidin Chloride with Potassium Hydroxide.**Isolation of Phloroglucin.*

About 1 gm. of powdered anthocyanidin chloride was stirred into 40 gm. of 75 per cent potassium hydroxide heated to 180°C. The mixture, which turned very black in color, was heated for a few minutes nearly to boiling. It was then diluted with 100 cc. of water, cooled, acidified with hydrochloric acid, and extracted with 5 portions of ether. The very dark colored ethereal solution was shaken with 100 cc. of 5 per cent sodium bicarbonate which removed practically all the coloring matter, leaving the ethereal layer nearly colorless. The ether was evaporated and the residue was dissolved in a little warm water, decolorized with norit, and the filtrate concentrated in a vacuum desiccator over sulfuric acid. The substance separated in nearly colorless crystals. After drying at 105° in a vacuum over phosphorus pentoxide, the substance melted at 216°C. (uncorrected).

The aqueous solution gave the following reactions: (a) Ferric chloride produced a purplish coloration; (b) a piece of pine wood, moistened with concentrated hydrochloric acid, assumed a deep red color; (c) Fehling's solution was reduced; (d) adding aniline nitrate and sodium nitrite gave a bright red precipitate; and (e) bromine water produced a cloudy solution from which colorless prisms crystallized.

The melting point and the above mentioned reactions indicate that the substance was phloroglucin.

The sodium bicarbonate solution mentioned above, should have contained the gallic acid, but we were unable to obtain this substance in crystalline form. The solution was acidified and extracted with ether. On evaporation of the ether a very dark colored residue that could not be completely decolorized was left. The aqueous solution was acid in reaction and it gave with ferric chloride a bluish black coloration; on standing, this solution deposited a black precipitate. The aqueous solution left a dark colored syrup on concentrating it in a vacuum, but no crystals separated.

Extraction of the Grape Pigment with Glacial Acetic Acid.

The grape skins were prepared and pressed in the same way as before. About 2 kilos of pressed grape skins were digested in 3 liters of glacial acetic acid, with occasional stirring, for 10 days. The material was then ground to a pulp in a mortar, filtered on Buchner funnels, and washed with 2 liters of glacial acetic acid. The filtrate measured about 5.6 liters and it was of a deep carmine-red color. The greater portion of the pigment was precipitated as a thick purplish colored gummy mass on adding 18 liters of ordinary ether. After the mixture had stood in the ice box over night the dark red-colored supernatant liquid was poured off and the residue was shaken with several portions of ether in order to remove the acetic acid as completely as possible.

The precipitated pigment was dissolved in 2 liters of 0.5 per cent hydrochloric acid. A small amount of nearly colorless insoluble material was removed by filtration. The intensely carmine-red filtrate was warmed on the water bath to 40°C. and it was then mixed with a solution containing 80 gm. of picric acid in 8 liters of warm water. The anthocyanin picrate separated slowly, after the solution had cooled, as bright red crystalline needles, generally arranged in bundles. After the crystals had been filtered, washed with ether, and dried, they weighed 8.6 gm.

To the filtrate, which was dark red in color, was added 1 liter of concentrated hydrochloric acid, and the solution was allowed to stand for 24 hours in the ice box. The dark red crystalline plates that separated were filtered, washed with dilute hydrochloric acid, and dried in a vacuum over sulfuric acid and potassium hydroxide. The dry substance, 55.6 gm., was digested in 400 cc. of warm benzene, filtered, washed with warm benzene and finally with ether, and dried in a vacuum over sulfuric acid. The dark red picrate weighed 2.7 gm.

The crude picrates were converted into anthocyanin chloride in the manner already described. The yield was 7.1 gm. This substance was purified and crystallized by the same method as before. The crystalline anthocyanin chloride after drying in the air weighed 4.15 gm.

It was analyzed after drying at 105° in a vacuum over phosphorus pentoxide.

0.1623 and 0.2383 gm. substance lost on drying 0.0117 and 0.0174 gm.

0.1506 gm. substance: 0.0645 gm. H_2O and 0.2895 gm. CO_2 . 0.2208 gm. substance: 0.0587 gm. AgCl .

Calculated for $\text{C}_{23}\text{H}_{25}\text{O}_{12}\text{Cl}$ (528.5). C 52.22, H 4.73, Cl 6.71 per cent.

Found. C 52.42, H 4.79, Cl 6.57 per cent.

Calculated for 2.5 H_2O , 7.84 per cent.

Found. H_2O 7.20, 7.30 per cent.

The anthocyanidin chloride was prepared by hydrolyzing the glucoside and it was purified by recrystallization in the manner already described. For analysis it was dried as mentioned above.

0.1246 and 0.1240 gm. substance lost on drying 0.0082 and 0.0079 gm.

0.1164 gm. substance: 0.0426 gm. H_2O and 0.2359 gm. CO_2 . 0.1160 gm. substance: 0.0422 gm. AgCl .

Calculated for $\text{C}_{17}\text{H}_{15}\text{O}_7\text{Cl}$ (366.5). C 55.66, H 4.09, Cl 9.68 per cent.

Found. C 55.27, H 4.09, Cl 9.00 per cent.

Calculated for 1.5 H_2O , 6.86 per cent.

Found. H_2O 6.58, 6.37 per cent.

Methoxyl determination. 0.2020 gm. substance: 0.1546 gm. AgI .

Calculated for 1 CH_3O , 8.45 per cent.

" " 2 CH_3O , 16.91 " "

Found. CH_3O 10.11 per cent.

It is evident from the above analyses that the pigments isolated from Norton grapes by the two methods were identical in composition. It should be noted, however, that the isolation of the anthocyanin by the dilute hydrochloric acid method is much easier, less expensive, and less time-consuming than by glacial acetic acid. The yield, by the first method, of crystalline picrate is also somewhat greater.

The Anthocyan in Concord Grapes (Vitis labrusca).

The skins of Concord grapes were prepared, pressed, and digested in glacial acetic acid. The pigment was isolated from the acetic acid solution, precipitated by means of picric acid, and the glucoside was prepared from the picrate as already described under Norton grapes. From 3,850 gm. of fresh pressed skins we obtained 14 gm. of the crude anthocyanin chloride.

Purification of the Glucoside.

3 gm. of the crude glucoside were dissolved in 20 cc. of methyl alcohol and to the bright carmine-red solution were added 25 cc.

of ethyl alcohol, containing 7 cc. of 21 per cent hydrochloric acid in absolute alcohol. The slight precipitate that formed on adding the acidified alcohol was filtered off and the clear filtrate was allowed to stand in a loosely covered dish at room temperature for 3 days. The substance separated slowly in the form of small dark reddish brown uniform granules. The substance was filtered and washed, first in a mixture of alcohol and ether, and finally in ether. After drying in the air the substance weighed 1.5 gm. It was analyzed after drying at 105° in a vacuum over phosphorus pentoxide.

0.1507 and 0.1941 gm. substance lost on drying 0.0089 and 0.0120 gm.

0.1418 gm. substance: 0.0619 gm. H₂O and 0.2708 gm. CO₂. 0.1812 gm. substance: 0.0455 gm. AgCl.

Calculated for C₂₃H₂₅O₁₂Cl (528.5). C 52.22, H 4.73, Cl 6.71 per cent.

Found. C 52.08, H 4.88, Cl 6.21 per cent.

Calculated for 2 H₂O, 6.37 per cent.

Found. H₂O 5.90, 6.18 per cent.

The composition of this substance is identical with that of the anthocyanin chloride that had been isolated from Norton grapes.

Preparation and Analysis of the Anthocyanin Picrate.

About 4 gm. of the crude glucoside were dissolved in 400 cc. of water, containing a few drops of hydrochloric acid. The solution was filtered and mixed with 2.5 liters of a saturated aqueous solution of picric acid warmed to 40°C. The picrate separated when the solution was allowed to stand at room temperature over night in the form of bright red bundles of needle-shaped crystals. The crystals were filtered on a Buchner funnel and washed carefully with ether. After drying in the air the substance weighed 2.2 gm. For analysis it was dried in a vacuum at 105° over phosphorus pentoxide.

0.1592 and 0.2385 gm. substance lost on drying 0.0137 and 0.0204 gm.

0.1455 gm. substance: 0.0489 gm. H₂O and 0.2583 gm. CO₂.

0.2179 gm. substance: N, 10.4 cc. at 20°C. and 747 mm.

Calculated for C₂₃H₂₅O₁₂·C₆H₂(NO₂)₃OH (722). C 48.20, H 3.87, N 5.81 per cent.

Found. C 48.41, H 3.76, N 5.46 per cent.

Calculated for 4 H₂O, 9.06 per cent.

Found. H₂O 8.60, 8.55 per cent.

The purified picrate mentioned above was converted into anthocyanin chloride by the method already described. It was analyzed after drying in the manner mentioned above.

0.1467 and 0.2086 gm. substance lost on drying 0.0099 and 0.0136 gm.

0.1368 gm. substance: 0.0586 gm. H_2O and 0.2645 gm. CO_2 . 0.1946 gm. substance: 0.0511 gm. AgCl .

Calculated for $\text{C}_{23}\text{H}_{25}\text{O}_{12}\text{Cl}$ (528.5). C 52.22, H 4.73, Cl 6.71 per cent.

Found. C 52.73, H 4.79, Cl 6.49 per cent.

Calculated for 2 H_2O , 6.37 per cent.

Found. H_2O 6.74, 6.51 per cent.

Hydrolysis of Anthocyanin Chloride.

Quantitative Determination of Anthocyanidin Chloride and Glucose.

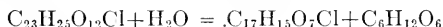
The dried, purified anthocyanin chloride, 0.9396 gm., was dissolved in 10 cc. of warm water and to this solution were added 50 cc. of 25 per cent hydrochloric acid. The solution was rapidly heated to boiling and boiled for 4 minutes. The sugar-free pigment began to crystallize out after the solution had boiled for 2 minutes. After the solution had cooled and stood in a freezing mixture for some time the precipitated pigment was filtered off, washed with cold 25 per cent hydrochloric acid, dried in a vacuum desiccator over sulfuric acid and potassium hydroxide, and finally dried in vacuum over phosphorus pentoxide at 105°C .

The filtrate from the anthocyanidin chloride was dark red in color. The pigment that remained dissolved in the dilute hydrochloric acid was extracted with amyl alcohol, leaving a colorless solution. The amyl alcohol was evaporated and the residue was dried as mentioned above.

The dry crystalline anthocyanidin chloride weighed 0.5627 gm. and the pigment extracted with amyl alcohol weighed 0.0513 gm. The total weight was, therefore, 0.6140 gm.

The glucose was estimated in the filtrate by Fehling's solution after first extracting the amyl alcohol with ether. The amount of glucose found was 0.2384 gm.

Calculated according to the equation



anthocyanidin chloride, 0.6301 gm.; glucose, 0.3095 gm. We recovered, therefore, 97.4 per cent of the anthocyanidin chloride, but only 77 per cent of the glucose.

Identification of the Glucose by means of the Osazone.

The filtrate from another portion of hydrolyzed glucoside was extracted with amyl alcohol. The excess of hydrochloric acid was removed by adding lead carbonate, filtering, and precipitating the filtrate with hydrogen sulfide. After removing the lead sulfide, the filtrate was concentrated under reduced pressure to a thin syrup. The syrup was dissolved in 15 cc. of warm water, filtered, mixed with phenylhydrazine reagent, and heated on the water bath. The osazone was recrystallized from warm dilute alcohol. The needle-shaped crystals that separated appeared to be identical in form, when examined under the microscope, with glucosazone prepared from pure dextrose. When rapidly heated in a capillary tube the dry osazone melted with decomposition at 204°C. (uncorrected). It is evident, therefore, that the sugar, liberated on hydrolyzing the glucoside, is dextrose.

Anthocyanidin Chloride from Concord Grapes.

The anthocyanidin chloride, obtained on hydrolyzing the glucoside from Concord grapes, appeared to crystallize less readily than the corresponding preparation obtained from Norton grapes. There was, however, no difference in composition. The substance was analyzed after drying at 105°C. in a vacuum over phosphorus pentoxide.

0.1594 and 0.2123 gm. substance lost on drying 0.0116 and 0.0151 gm.

0.1478 gm. substance: 0.0518 gm. H_2O and 0.3035 gm. CO_2 . 0.1971 gm. substance: 0.0727 gm. AgCl .

Calculated for $\text{C}_{17}\text{H}_{13}\text{O}_7\text{Cl}$ (366.5). C 55.66, H 4.09, Cl 9.68 per cent.

Found. C 56.00, H 3.92, Cl 9.12 per cent.

Calculated for 1.5 H_2O , 6.86 per cent.

Found. H_2O 7.27, 7.11 per cent.

SUMMARY.

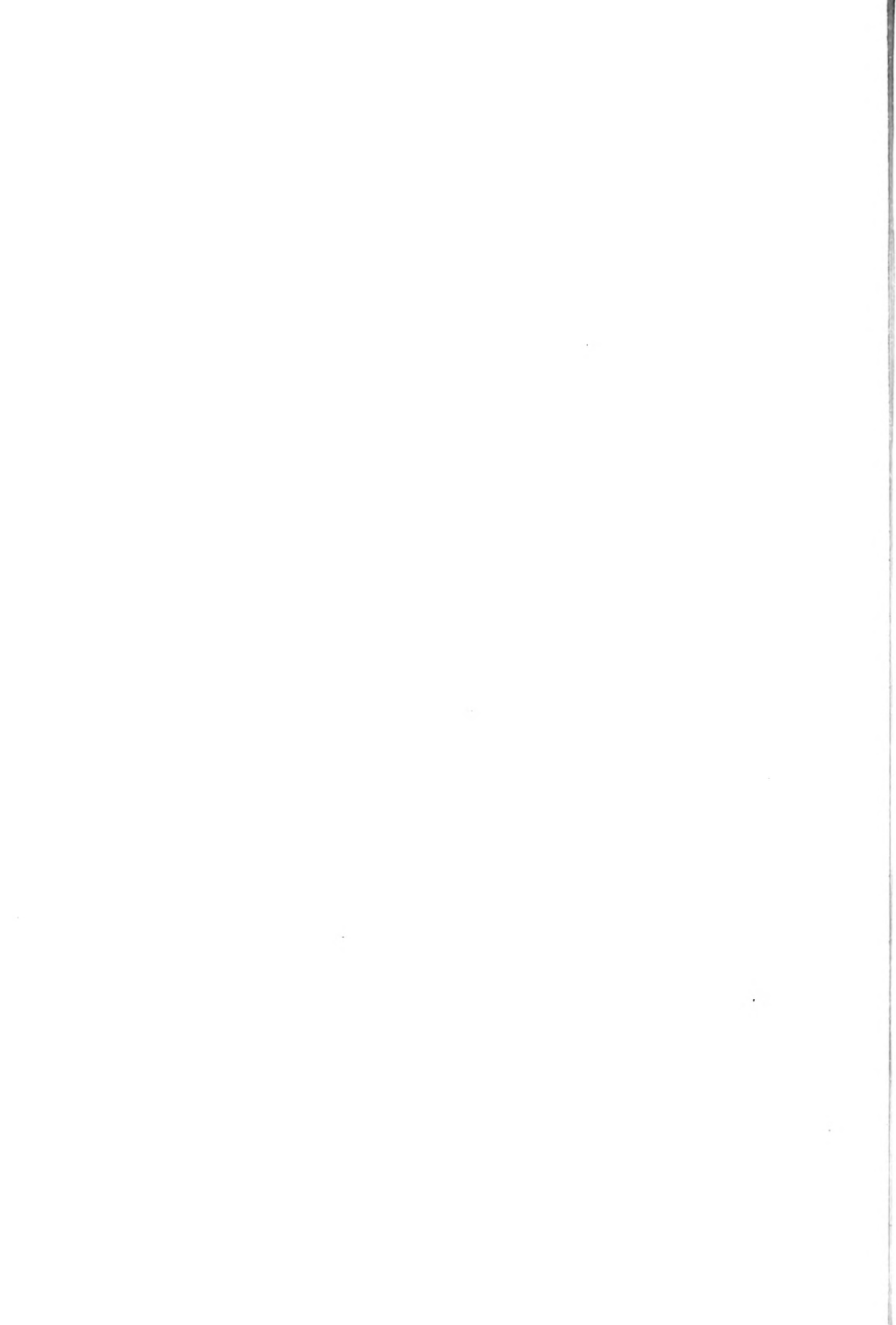
The pigments occurring in Norton and Concord grapes have been isolated and studied. After purification these pigments were found to be identical in composition and properties.

The coloring matter consists principally of a monoglucoside, anthocyanin, which is very similar to oenin derived from *Vitis vinifera*, differing from the latter only in the color reaction with ferric chloride.

The anthocyanin chloride corresponds to the formula $C_{23}H_{25}O_{12}Cl$. On hydrolysis with boiling hydrochloric acid it yields 1 molecule of glucose and 1 molecule of the sugar-free pigment, anthocyanidin chloride, $C_{17}H_{15}O_7Cl$.

The spectrum of anthocyanin chloride consists of one broad band with indefinite margins extending from the yellow into the blue.

The anthocyanidin chloride crystallizes in beautiful prisms. It is very similar to oenidin chloride which is obtained on hydrolyzing oenin, but it differs in that it contains a lower percentage of methoxyl groups.



STUDIES OF URINARY ACIDITY.

II. THE INCREASED ACIDITY PRODUCED BY EATING PRUNES AND CRANBERRIES.

BY N. R. BLATHERWICK AND M. LOUISA LONG.

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(Received for publication, July 31, 1923.)

Practically every one now understands that most of the various organic acids present in the fruits are completely oxidized within the body. The contained acids, therefore, do not exhibit any ultimate acid effect but, on the contrary, fruits cause the production of less acid urines because of the alkaline ash which they yield. Prunes, plums, and cranberries are an exception to this general truth, as was demonstrated by one of us (Blatherwick, 1914). These fruits contain benzoic acid and some other substance or substances from which the body forms hippuric acid which is eliminated in the urine. However, the magnitude of the effects on urinary acidity found in the investigation referred to seemed too large to be explained by the synthesis of hippuric acid from the small amount of benzoic acid said to be present in these fruits. We have, therefore, repeated this experiment, making determinations of hippuric acid, the values for which were not obtained in the former investigation.

Two healthy young individuals volunteered as subjects for this study. Subject B. was given a uniform basal diet of the following composition: whole milk, 1,200 cc.; graham crackers, 300 gm.; one egg; raw apple, 150 gm.; and cream, 200 cc. The diet of Subject L. was similar excepting that she ate neither the egg nor the cream. After several days on this diet the fruit in question was added to the diet. The prunes and cranberries were eaten in the form of a sauce and were weighed before cooking.

The methods used were those described in the first paper of this series (Blatherwick and Long, 1922). Hippuric acid was determined by the method of Kingsbury and Swanson (1921).

The experimental data obtained from this study are given in Table I. A careful perusal of the values for the urines of the

TABLE I.
Composition of Urine.

Date.	Volume.	pH	Titrate-ability 0.1 N.	P	Organic acids 0.1 N.	Hippuric acid.	Ammonia N.	N	Remarks.
Subject B.									
1922	cc.		cc. gm.	cc.	gm.	gm.	gm.		
Mar. 14	1,045 6.3		231 0.95	473	0.93 0.27	9.09			Basal diet.
" 15	915 6.2		306 1.24	452	0.83 0.25	9.42			" "
" 16	1,105 6.3		269 1.15	523	0.89 0.22	9.61			" "
" 17	1,035 6.5		189 1.08	522	0.87 0.19	9.11			" "
" 18	980 6.5		239 1.10	557	1.00 0.20	9.37			" "
" 21	1,110 5.7		395 1.25	924		0.37 8.96			" " + 300 gm.
" 22	915 6.3		292 1.14	822	7.50 0.26	7.67			prunes.
" 23	957 6.0		399 1.28	962	9.58 0.36	8.52			
" 24	910 5.8		329 1.03	992	11.24 0.33	6.73			
" 25	1,120 6.0		389 1.26	1,197	10.14 0.41	7.71			Basal diet + 450 gm. prunes.
Subject L.									
Mar. 14	900 6.3		228 0.85	472	0.68 0.19	7.92			Basal diet.
" 16	1,010 6.6		180 1.02	507	0.85 0.21	9.09			" "
" 17	1,190 6.6		218 0.92	449	0.75 0.15	9.12			" "
" 18	1,010 6.4		227 1.05	452	0.82 0.16	9.26			" "
" 21	1,230 5.1		381 0.85	904		0.39 7.69			" " + 300 gm.
" 22	1,100 5.3		372 0.92	870	8.88 0.30	7.37			prunes.
" 23	1,110 5.3		351 0.90	988	9.22 0.35	6.44			
" 24	1,160 5.7		316 1.17	1,086	12.32 0.31	6.26			
" 7	940 5.3		311 0.89	624	4.74				Basal diet + 305 gm. cranberries.

prune period shows that the following changes occurred: decreases in the pH value (a greater concentration of hydrogen ions); in-

creases in the titratable acidity; no significant changes in the phosphorus excretion; significant increases in the ammonia output; marked decreases in the total nitrogen; and very large increases in the values for organic acids and for hippuric acid.

These results demonstrate that the increased acidity of the urine which is produced by eating prunes is in fact due to the synthesis and excretion of hippuric acid. The augmented values for organic acids are found to agree quite closely with the theoretical figures calculated from the extra excretion of hippuric acid. Thus, the average daily organic acid excretion of Subject B. on the basal diet was 505 cc. of 0.1 N acid, and the average daily output of hippuric acid was 0.90 gm. On March 24, 11.24 gm. of hippuric acid were eliminated, an increase of 10.34 gm. over the preliminary period. Since hippuric acid is titrated to the extent of about 90 per cent by the method used, therefore, 10.34 gm. \times 0.90 = 9.31 gm. should have been available for titration, or the equivalent of $\frac{9.31}{0.0179} = 520$ cc. of 0.1 N acid. The ob-

served increase of organic acid excretion over that of the preliminary period was 487 cc. This calculation leaves a discrepancy of only 37 cc. of 0.1 N acid. There was considerable variation from day to day but it is evident that the two methods are reliable. There was no increase in the excretion of phosphorus such as occurs when sour milk is drunk (Blatherwick and Long, 1922). It will be noted that the prune diet produced a marked fall in the output of total nitrogen. Since there was no evidence of greater elimination from the bowel, it is probable that this represents a protein-sparing effect of the carbohydrate contained in the fruit. The same drop in total nitrogen was observed in the original investigation (Blatherwick, 1914).

On March 7, Subject L. ate 305 gm. of cranberries. The changes produced in the composition of the urine were similar to those which followed the ingestion of prunes, but the extra hippuric acid amounted to less than one-half that formed from the same weight of prunes.

What is the source of the hippuric acid formed when these fruits are metabolized? Radin (1914) reported that prunes and cranberries contain, respectively, 0.05 and 0.06 per cent of benzoic acid. We have made determinations of the benzoic acid

content of these fruits, using the following method. Approximately 50 gm. of the fruit were boiled until disintegrated in water containing about 6 gm. of sodium hydroxide. The digest was then made to 500 cc. and filtered. 100 cc. of filtrate were transferred to a distilling flask and 10 cc. of concentrated sulfuric acid were added. Distillation in a current of steam was then conducted until from 400 to 500 cc. had collected. The distillate was next concentrated to a volume of about 25 cc., transferred to a Kjeldahl flask, and about 7 gm. of sodium hydroxide were added. The remainder of the procedure was similar to that used for the determination of hippuric acid in urine. This method gave values of 0.147 per cent benzoic acid in prunes and of 0.096 per cent in cranberries. The extra elimination of hippuric acid by Subject B. on March 24 was 10.34 gm. Supposing this to have arisen from benzoic acid *per se* would require a content of 2.35 per cent in the prunes. Such calculations compel one to look elsewhere for the chief precursors of hippuric acid in these fruits. Quinic acid is the most probable compound concerned in this synthesis.

SUMMARY.

Further data are presented which show that the increased acidity of urines formed after eating prunes and cranberries is produced by hippuric acid. The greater portion of this hippuric acid is derived from sources other than benzoic acid.

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STUDIES IN THE CHEMISTRY OF HEMOGLOBIN.

I. THE PREPARATION OF HEMOGLOBIN.

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Most of the physicochemical problems in the blood appear to center about hemoglobin, largely on account of the peculiar properties which depend on its combination with oxygen. We have therefore attempted to devise a more satisfactory method for the preparation of pure hemoglobin on which we could base further studies.

There have in the past been many such attempts. Beginning with the observation of Hünefeld (1) in 1840 that the blood of an earthworm yielded a crystalline substance, Reichert (2), von Kölliker (3), Leydig (4), Budge (5), Kunde (6), and many others noted that blood from various species yielded a similar crystalline substance. As early as 1852 Funke (7) described the method of laking blood with water and then inducing crystal formation with alcohol and ether. Although he prepared only small quantities of hemoglobin, the principle of this method has been widely used up to the present.

In 1862 Hoppe-Seyler (8) noted the characteristic absorption spectra of various modifications of hemoglobin. His observation that hemoglobin changed readily when in acid solution is significant, as is the further observation that all the hemoglobin, under ordinary conditions, never goes into the changed form that we now recognize as methemoglobin, thereby suggesting that met- and oxyhemoglobin form an equilibrium mixture. Whereas the experiments of Hoppe-Seyler suggested the respiratory nature of the pigment, Stokes (9) apparently deserves the credit for appreciating its highly important function in the blood. The important fact that oxyhemoglobin is less soluble than the reduced form was noted by Kühne (10) as early as 1865.

One may, however, consider that serious attention to the preparation of hemoglobin on other than a microscopic scale began at the time of Preyer

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(11) (in 1860 or thereabouts) who collected most of the knowledge then current in his book. The methods of purification described by him all depend on crystallization. The numerous modifications may be separated into three general groups. Frequently two methods were combined in order to obtain a better yield.

First Group.—An impure solution of hemoglobin was obtained from corpuscles or blood clot and laked either by alternate freezing and thawing or by the addition of water, ether, or bile salts. To this solution were added ether, in the proportion of 50 to 70 cc. per liter, and alcohol to give a concentration of about 25 per cent by volume. The resulting solution was set aside in the cold until a good crop of crystals appeared. The crystalline yield could be dissolved in water and then recrystallized as desired in a similar manner.

Second Group.—An impure solution of hemoglobin prepared as in the first method was treated with ammonium sulfate sufficient to give approximately half saturation or sodium sulfate in high concentration. Crystals appeared after standing at room temperature for varying periods of time. After solution in water, recrystallization could be repeated.

Third Group.—A rather more concentrated but impure solution of hemoglobin was treated with oxygen and carbon dioxide. The crystals obtained could be dissolved in a minimal amount of water, and again formed by passing oxygen and carbon dioxide into the solution. Recently, Dudley and Evans (12) have obtained crystals from horse blood by the use of oxygen alone. In our experience this method is not very successful. It has been improved by Heidelberger and made extremely useful (13).

Except from the method last described the product was usually unstable and never pure. The alcohol-ether method has the further objection that proteins are often denatured under such conditions, as well as the possibility that a loose lipid-protein complex may be destroyed. Unfortunately, the use of ammonium sulfate results in a relatively high proportion of methemoglobin. Consequently, most investigators have contented themselves with dialyzed corpuscles, which, to be sure, have led to significant results, especially in the hands of Barcroft (14), Haldane, and their collaborators. Unfortunately, such results are not always reproducible.

Bearing the older procedures in mind, we sought to develop a method which involved only physical processes and the addition of such substances as were already present in the blood. Viewed in this way, the preparation of a pure product evidently resolved itself into three distinct problems: freeing the hemoglobin from plasma proteins; of stromata and intracorpuseular protein; and of electrolytes.

In our procedure corpuscles from citrated horse blood¹ were

¹ Obtained from the Antitoxin Laboratory of the Massachusetts Department of Public Health through the courtesy of its director, Dr. G. Benjamin White. In this way we were assured of clean material at the outset.

washed in the ordinary laboratory centrifuge six times with an equal volume of 1.5 per cent sodium chloride solution. A series of experiments showed that a concentration of 1.5 per cent sodium chloride was most advantageous in that the corpuscles sedimented promptly, were closely packed by centrifugation, and were very little hemolyzed. Higher concentrations apparently decreased the life of the corpuscles and were therefore less desirable in spite of better packing. Heating the supernatant fluid after the sixth washing showed that very little plasma protein remained. Further washing probably removed more plasma protein, but increasing hemolysis rendered heat coagulation inapplicable as a test. Consequently, since the amount of residual plasma protein must necessarily have been very small, we washed only six times.

At this point we employed a different method of centrifugation. By means of the Sharples centrifuge, an apparatus consisting essentially of a hollow cylinder of about 2 inches diameter which rotates 25,000 to 35,000 R.P.M., we could secure an extremely concentrated buttermilk paste composed largely of crystalline hemoglobin and shrunken corpuscles. This method of centrifugation was extremely effective since the high speed attained results in greater centrifugal force than that attained in centrifuges of greater radii running at slower speeds. It had the further advantage that the process was continuous, inasmuch as a suspension of corpuscles diluted with 5 to 6 volumes of 1.5 per cent sodium chloride solution could be run into the machine while the paste and waste solution appeared at the respective outlets. As a result we could use relatively large volumes thereby greatly increasing the effectiveness of our final washing. The high pressure developed in this method destroyed the corpuscles and liberated hemoglobin. Reference to Table I will indicate the hemoglobin concentration attained by this procedure. It is worthy of note that the paste contained about 50 per cent hemoglobin by weight, using either the nitrogen content or oxygen capacity of diluted samples as an index of concentration.

The paste from the Sharples centrifuge was next diluted with three to four times its own volume of water. Nearly complete hemolysis resulted. Acting on the observation of Adair, Barcroft, and Bock (15) that the addition of sodium chloride to dialyzed corpuscles causes the previously difficultly visible ghosts to become

readily visible under the microscope, we added sufficient sodium chloride by weight to give a 2 per cent solution. Centrifuging again at this point, in the Sharples centrifuge, rid the solution of nearly all the stroma substance and gave a moderately concentrated solution of hemoglobin containing very small amounts of foreign protein as an impurity. This step had the added virtue of eliminating bacteria that may have been present.

This solution was next dialyzed in collodion membranes prepared in Erlenmeyer flasks of 250 to 500 cc. capacity from a solution made up as follows:

Pyroxylin.....	5 gm.
Ether.....	75 cc.
Ethyl alcohol.....	25 "
" acetate.....	5 "

a modification of the preparation suggested by Looney (16). This type of membrane was selected since it gave a high permeability to electrolytes with very slight leakage of hemoglobin—as was demonstrated by a series of pertinent experiments. Dialysis was usually continued against frequent changes of distilled water until the dialysate gave no precipitate with silver nitrate in acid solution and until the filtrate from a coagulated portion of the hemoglobin within the membrane gave at most a faint cloud with silver nitrate in acid solution. This type of dialysis lasted about 3 days. Occasionally the membranes contained a small amount of reddish flocculent precipitate, presumably due to precipitation of traces of serum globulin or of stroma protein that had not previously been removed. Whenever this occurred, the solution was again centrifuged. The solution always had the classical clear burgundy-red color characteristic of oxyhemoglobin.

Dilution always occurred during the course of this preliminary dialysis. Since we felt that the usual forms of drying might give methemoglobin or otherwise denature our product, we adopted as a final step the method of negative pressure dialysis described by Sørensen (17) as slightly modified by Cohn.² In this method the osmotic pressure of the dialyzing solution was kept constant. Hence, as the electrolytes passed into the dialysate, the protein solution was concentrated. In this way we frequently halved the

² Cohn, E. J., Personal communication.

volume of our solution over night and invariably obtained a deposit of crystalline hemoglobin at the bottom of our membranes in the course of 2 to 3 days. Solution and recrystallization were then possible, but up to the present have not been utilized. The contents of the membranes were then used directly or in appropriate dilutions in the work that follows.

The entire process with the exception of centrifugation was carried out in the cold room at 2°C. Centrifugation was performed in iced containers.

Hemoglobin prepared in this manner gives clear burgundy-red solutions. The crystals were always readily soluble in distilled water. It is noteworthy that preparations obtained in this manner were always of nearly the same concentration; they were in fact saturated solutions of hemoglobin at the temperature of the cold room. The hydrogen ion concentration of the reduced solution as measured by the hydrogen electrode never varied more than 0.3 from $1 \times 10^{-7}M$. It is probable that our solutions were not isohydric with the isoelectric point of hemoglobin. They were reducible by the usual procedures and took up oxygen with avidity. No diminution of oxygen capacity occurred until dialysis was begun. It is possible, as other experiments—not yet complete—suggest, that this may in part be due to the removal of base from hemoglobin during dialysis and the resulting formation of the acid form which, with slighter dissociation, has a lower oxygen capacity. This hypothesis accords with results of Adolph and Ferry (18) who noted that hemoglobin in equilibrium with oxygen tensions sufficient to give approximately half saturation took up more oxygen after addition of alkali, but less after addition of acid.

In Tables I and II are given the results of analyses indicating the purity of our product. Judging from the figures for ash and chlorides, our electrolyte content was extremely low. It is believed that comparison of values for the concentration of hemoglobin derived from nitrogen analyses performed by the Kjeldahl method with those given by the method of oxygen capacity using the Van Slyke constant volume apparatus is a valuable addition to the technique, furnishing as it does a criterion for the presence of serum and stroma proteins as well as of methemoglobin.

The method of cataphoresis was used as a test for freedom from other proteins. This depends on the fact that when proteins are

TABLE I.

Hb No.	Date.	Experiment No.	Dilution.	N in 1 cc.	PO ₂ combined (atmospheres).	O ₂ combined.	Molar concentration of Hb as diluted.		Hb by O ₂ capacity Hb by N.	Remarks.
							By O ₂ capacity.	By N.		
Hb XI. During preparation.										
XI	1922					<i>vol. per cent</i>				
	May 12	1	1:5	11.84	0.2	8.81	0.00393	0.00408	0.963	Diluted corpuscular cream.
	" 12	2	1:2	5.76	0.2	4.45	0.00198	0.00199	0.995	Washed red blood cells before Sharples.
	" 12	3	1:18.12	5.23	0.2	4.16	0.00186	0.00180	1.033	Sharples cream.
	" 12	4	1:2	14.83	0.2	11.23	0.00501	0.00511	0.980	Stroma-free solution.
	" 13	5	1:1		0.2	5.54	0.00248	0.00256	0.969	"
XIa XIb	" 19	6			0.2	7.46	0.00333			Before pressure dialysis.
	May 22	310	5:7	18.73*	1.0	8.70	0.00388	0.00461†	0.842‡	After pressure dialysis.
	" 25	312	5:6	18.12*	1.0	10.78	0.00481	0.00520†	0.925‡	"

* Undiluted.

† Corrected value for dilution.

‡ These were samples from two separate pressure dialysers which had been running for different lengths of time.

TABLE II.

Hb No.	Date.	Experiment No.	Dilution.	Molar concentration of Hb in diluted sample.		Molar concentration of Hb in material.		Relative concentration of Hb referred to blood as 0.01 M.	Remarks.
				By O ₂ capacity.	By N.	By O ₂ .	By N.		
Hb XI. During preparation.									
XI	¹⁹²² May 12	1	1:5	0.00393	0.00408	0.01965	0.02040	2.0	Diluted corpuscular cream.
	" 12	2	1:2	0.00198	0.00199	0.00396	0.00398	0.4	Washed red blood cells before Sharples.
	" 12	3	1:18, 12	0.00186	0.00180	0.03370	0.03262	3.3	Sharples cream.
	" 12	4	1:2	0.00501	0.00511	0.01002	0.01022	1.0	Stroma-free solution.
	" 13	5	1:4	0.00248	0.00256	0.00992	0.01024	1.0	"
	" 19	6		0.00333		0.00333		0.33	Before pressure dialysis.
XIa	" 22	310	5:7	0.00388	0.00461	0.00543	0.00646	0.6	Final product.
XIb	" 25	312	5:6	0.00481	0.00520	0.00577	0.00625	0.6	"

subjected to a difference in electric potential they migrate to the cathode if they are dissociated as bases at reactions more acid than their isoelectric points; to the anode if dissociated as acids at less acid, or at alkaline, reactions. Inasmuch as the isoelectric points of the serum proteins—serum albumin and serum globulin, which coincide with pH 4.7 and 5.4, respectively—differ widely from that of hemoglobin at pH 6.78, it is possible to adjust the reaction to such a point that when a current is passed hemoglobin will migrate in one direction, the serum proteins in another. We added to the hemoglobin preparation sufficient acid to bring the reaction to pH 6.5 to 6.2. This in turn was mixed with a phosphate solution as buffer, after which the adjusted solution was delivered into a modified U-tube apparatus, previously described by Cohn, Gross, and Johnson (19). The current was turned on for 24 hours. At the end of this time hemoglobin ionized as base had migrated towards the cathode; the other blood proteins behaved as acid radicals and moved towards the anode. The anodal portion of the fluid was removed, enough potassium sulfate added to insure complete coagulation, and then heated as a test for foreign blood protein. In our best preparations this test showed only very small amounts of foreign blood protein contained in the clear colorless fluid from the anodal portion of the vessel. The white flocculent coagula obtained in this way were readily distinguishable from the characteristic chocolate-brown hemoglobin coagulum. This procedure can readily be made quantitative, and it is believed that it is a valuable step in effecting protein separations, as well as determining the freedom of one protein from another.

Using material prepared in this manner we have, with E. J. Cohn, determined a titration curve of reduced hemoglobin with the hydrogen electrode. These results are reproducible. But as a result of the work of J. B. Conant (20) upon the relationship of met- and reduced hemoglobin, we consider that the meaning of such a titration curve is doubtful and are, therefore, unwilling to publish these data at this time. The implications of Conant's work concerning the preparation of hemoglobin free from methemoglobin are clearly understood and will shortly be made the subject of further investigation. We have, however, redetermined the isoelectric point of reduced hemoglobin by the method of cataphoresis in heavily buffered solutions. Our observations confirm those of

Michaelis (21) and show that the isoelectric point coincides with pH 6.78 ± 0.03 (see Table III). Further studies on the combination of hemoglobin with oxygen that are being conducted in this laboratory are also consistent with each other.

Consequently, we feel that while the method of preparation described is in no sense final, the results of our analyses and the reproducibility of our results demonstrate its usefulness.

TABLE III.
Cataphoresis of Reduced Hemoglobin.

Date.	pH	M/15 KH_2PO_4 cc.	M/15 Na_2HPO_4 cc.	Mixture of hemo- globin and buffer.	Migration towards		Remarks.
					Cathode.	Anode.	
1921		cc.	cc.				
July 2	6.70	56.25	43.75	Equal parts.	++	0	Marked migration.
" 9	6.72	55.50	44.50	" "	+	0	" "
" 7	6.75	53.25	46.75	" "	\pm	0	Slight, if any, migration.
" 18	6.78	52.00	48.00	" "	\pm	\pm	Doubtful migration both ways.
June 30	6.81	50.00	50.00	" "	0	+	Rose 4.3 mm. toward anode.
" 21	7.00	38.75	61.25	" "	0	++	Marked migration.

SUMMARY.

1. A method of preparation for pure hemoglobin is described. In it crystallization has been induced without recourse to chemical means, but solely by the aid of the Sharples centrifuge and the principle of dialysis of reduced pressure. A method for determining the freedom of proteins from each other and their separation is also described.

2. A comparison of values for the concentration of hemoglobin obtained from nitrogen analyses by the Kjeldahl method with those determined by the oxygen capacity method was applied as

a criterion for the presence of serum and stroma proteins as well as for the presence of methemoglobin.

3. The method of cataphoresis was used in determining the freedom of one protein from another.

4. The isoelectric point of reduced hemoglobin has been redetermined and coincides with pH 6.78.

We wish to thank Miss Jessie L. Hendry for the chloride, nitrogen, and ash analyses; and Dr. R. Carrasco-Formiguera for the oxygen capacity analyses as done with the Van Slyke constant volume blood gas apparatus.

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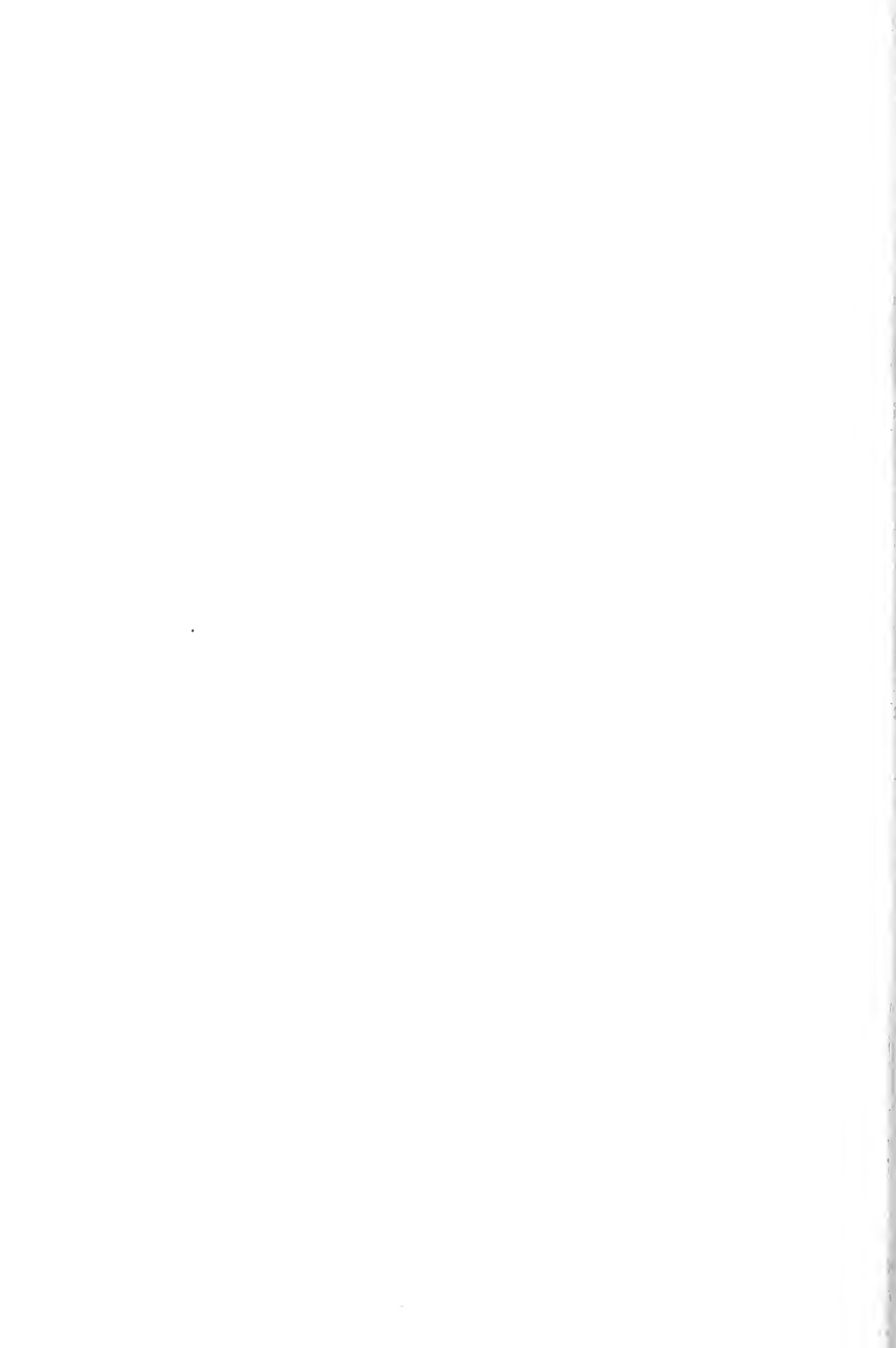
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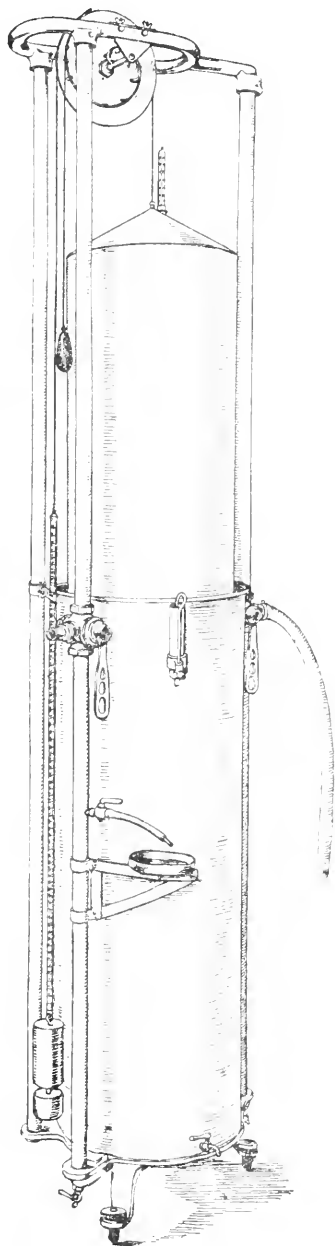
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AN OUTFIT

FOR

Basal Metabolism Estimations

AS DESIGNED BY

Dr. Cameron Vernon Bailey of the
Laboratory of Pathological Chemistry
New York Post Graduate Medical
School and Hospital

For detailed description see September issue
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BACTERIOLOGY

By

H. W. CONN, Ph.D.

Formerly Professor of Biology at Wesleyan University and Bacteriologist of the State Board of Health of Connecticut

AND

HAROLD J. CONN, Ph.D.

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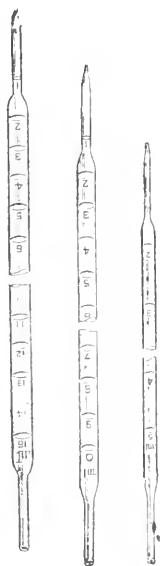
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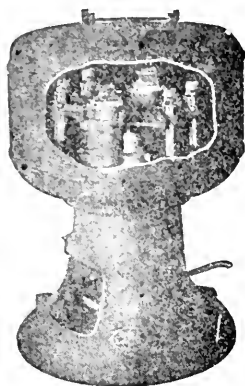
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